Studies on Adventitious Root Formation in *Pinus radiata*: Biochemical and Molecular Aspects

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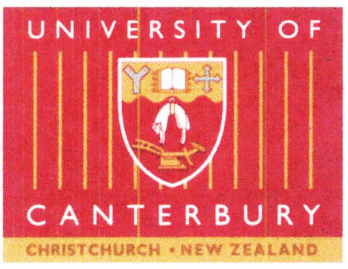

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In memory of my father, Li Sen (1924-1997)

Abstract

A reliable *in vitro* rooting system was established for further studies on biochemical and molecular aspects during adventitious root formation in *Pinus radiata* hypocotyls. High root counts and rooting efficiency were observed in a medium comprising ½ strength of the Murashige & Skoog's basal medium (1962), 20 g/l sucrose, 9 mg/l IBA and solidified with 8 g/l agar. In this system, the first cell division, formation of root primordium initial (a cluster of meristematic cells), well-organized root primordium (a dome shaped structure) and root emergence were typically observed at around day 4, 7, 10 and 13, respectively.

The increase of buffer soluble total proteins resulted in a peak at day 7 in the treatment with IBA (rooting treatment), but this peak was not observed in the other non-rooting treatments (including IBA+kinetin, kinetin alone and hormone-free control). Rooting related proteins were not observed using one-dimensional SDS polyacrylamide gel electrophoresis (PAGE). However, 19 proteins associated with the IBA treatment were observed by two-dimensional PAGE although their magnitudes were very small so that it was difficult to quantify them and establish them as novel proteins. Changes of peroxidase (PO), IAA-oxidase (IAA-O), amylase (Amy) and succinic dehydrogenase (SDH) activities assayed using tissue extracts were not significantly different between the rooting and the non-rooting treatments during the experiment. By contrast, the increase of polyphenol oxidase (PPO) activity was more significant at day 7 in the rooting treatment than in the other treatments. Nevertheless, histochemical localization of PO, PPO and SDH indicated that these enzymes were somehow associated with adventitious root formation. In the IBA treatment, the increase of starch content in the rooting region reached its maximum at

day 4 and 7. This increase coincided with the histological observation of accumulation of starch grains at the sites where root primordia initiated.

To identify the molecular signals that initiate adventitious root formation, subtracted cDNA library was made from IBA-treated hypocotyls following polymerase chain reaction (PCR) amplification of subtracted single strand-cDNAs. Inserts of 5 bacterial clones (R1-72, R2-7, R2-22, R2-35 and R3-24) were digoxigenin-labelled and used to detect their corresponding transcripts using RNA dot blotting. Using RNA dot blotting analysis, R1-72 detected an equivalent level of transcript in the hypocotyls at day 0 and day 7 in the treatments with and without IBA; whereas the other 4 clones, especially R2-7 and R2-35, detected different levels of transcripts in the hypocotyls during adventitious root initiation. Southern blotting analysis indicated that the R2-7 transcript is likely to be represented by a single gene or a member of a smaller gene family.

Adventitious root formation in *P. radiata* was significantly improved using *Agrobacterium rhizogenes*. Compared with the strain A4T, strain LBA9402 was very effective in increasing rooting percentage and root number. High rooting percentages and root numbers were obtained in the trials with hypocotyl segments, intact seedlings and adventitious shoots.

The findings in this research can assist further studies to shed some light on why some clonal cuttings of radiata pine root more easily than others do, and why mature cuttings are difficult for adventitious root induction.

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Abbreviations

1D	one dimensional
2,4-D	2,4-dichlorophenoxyacetic acid
2D	two dimensional
Amy	amylase
BA	6-benzyladenine
cDNA	complimentary deoxyribose nucleic acid
CHAPS	3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate
CTAB	cetyl triethylammonium bromide
$d(G)_n$	oligo deoxyguanosine (n residues long)
$d(T)_n$	oligo deoxythymidine (n residues long)
dATP	2'-deoxyadenosine-5'-triphosphate
DCP	2,4-dichlorophenol
dCTP	2'-deoxy-cytidinie-5'-triphosphate
d H_2O	distilled water
DDT	dithiothreitol
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine-5'-triphosphate
DIG	digoxigenin
DMF	dimethylformamide

DMSO dimethylsulphoxide

dNTP deoxynucleoside triphosphate

DOPA DL-3,4-dihydroxydiphenylalanine

ds double strand

dTTP thymidine-5'-triphosphate

EDTA ethylenediaminetetraacetic acid (sodium salt)

EGTA ethyleneglycol-O,O'-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

GD Gresshoff and Doy

GUS β-glucuronidase

HEPES N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid

IAA indole-3-acetic acid

IAA-O IAA oxidase

IBA indole-3-butyric acid

IEF isoelectric focusing

mRNA messenger ribonucleic acid

MS Murashige and Skoog

NAA naphthylacetic acid

PAGE polyacrylamide gel electrophoresis

PBA 2-phenoxy-2-methylpropionic acid

PCIB 2-(p-chlorophenoxy)-2-methylpropionic acid (or: p-chlorophenoxyisobutyric acid)

PCR polymerase chain reaction

PDA piperazine di-acrylamide

PO peroxidase

POAA phenoxyacetic acid

PPO	polyphenol oxidase
RNase	ribonulease
rRNA	ribosomal ribonucleic acid
SDH	succinic dehydrogenase
SDS	sodium dodecyl sulphate
ss	single strand
TEMED	N,N,N',N'-tetramethylethylenedimine
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopranoside

Chapter 1
General Introduction

1.1 Adventitious root formation

Adventitious root is a shoot-borne root whose origin is self-defining and can be traced back to the differentiation from cells within a shoot tissue (Esau, 1953; Barlow, 1986 1994; Lovell & White, 1986). Adventitious roots are common structural features of the angiosperms.

1.1.1 Importance

Occurring naturally on the shoot system above ground, the adventitious roots of some species have had opportunity to develop a structure and function somewhat different from those of the underground primary root system (Barlow, 1986). For example, they could absorb water and mineral nutrients from a location other than the soil, mechanically support shoot systems, climb or scramble over neighbouring plants and the terrain, and synthesise certain specialized metabolites such as cytokinins and amino acids needed for the growth of nearby shoot tissues (Barlow, 1986).

In plants there are two basic types of propagation, sexual (by seeds) and asexual (by vegetative organs). The importance of adventitious root formation, however, lies in asexual or vegetative propagation by cuttings (normally from stem and leaf), in which adventitious root formation gives rise to new plants. Vegetative reproduction makes it possible to produce many genetically identical individuals, which can all be derived from a single plant (Thulin & Faulds, 1968; Hammett, 1992). Some plant species produce no viable seeds (e.g. certain bananas and oranges), and these have to be propagated asexually (Hammett, 1992). In addition, vegetative reproduction

allows many plants that possess unique or valuable traits to be preserved and multiplied quickly.

1.1.2 Capacity for adventitious root formation

The capacity of adventitious root formation varies tremendously between plant species, between cultivars, with age and the nature of the organ or part. The most basic groups are plants that will root (Group I) and those that will not (Group II) after any rooting treatments (Lovell & White, 1986). Of those that will root two subgroups can be divided. In Subgroup I, the plants, such as brittle willow (Haissig, 1970) and Lombardy poplar (Shapiro, 1958), have their root primordial initials already present in stems but halt at some intermediate stages. In this case, to form roots it is only necessary to insert the stem cuttings into moist soil or water to induce their dormant root primordia into activity and growth (Wright, 1973). This kind of primordia have been termed as pre-formed primordia, pre-existing root initials, latent adventitious root primordia, presumptive primordial initials or potential primordial initials by different authors (Lovell & White, 1986). Lovell & white (1986) thought that presumptive primordial initials or potential primordial initials are more appropriate because the process of the primordia initiation was halted before organization and therefore the development lies in undeterminate.

Such plants, however, are exceptional and with majority of plants adventitious roots have to be induced, in which the inducers include wound and various chemicals. These plants belong to Subgroup II, in which the plants can subsequently be divided into "easy-to-root", "moderate-rooting" and "difficult-to-root" species according to the potential of adventitious root formation (Avidan & Lavee, 1978; Wiesman & Lavee, 1994).

Cuttings from some species, for example many herbaceous species, can form adventitious roots easily without the need for special treatment, although they do not belong to the range of so called "preformed primordia" species. In contrast, for some species adventitious roots will only be formed from cuttings treated with growth regulators. On the other hand, rooting capacity of some species may be very different from juvenile to mature phase, especially for woody plants (Lovell & White, 1986;

Hachett, 1988; Hackett & Murray, 1993). In this case, adventitious roots can be formed quickly and easily when cuttings are taken from young plants, while it is very difficult to root when cuttings are taken from older plants.

1.2 Some aspects associated with adventitious rooting

There are two possibilities that a cutting responds to a rooting treatment. It may or may not root. The former presents several alternative pathways which depends on different plants, organs and its environments (Lovell & White, 1986). Many events, including morphological, physiological, histological and biochemical changes occur from the excision of the cutting to root emergence. Information about the following aspects of adventitious root formation is summarized here.

1.2.1 Histological aspect

On the basis of histological changes during adventitious root formation, the rooting process may be divided into several stages (Mitsuhashi-Kato *et al.*, 1978a; Druart *et al.*, 1982; Hartmann & Kester, 1983; White & Lovell, 1984; De Klerk *et al.*, 1995). Nevertheless, there has been no general agreement as to the number and nature of the stages and also in the terminology used (Lovell & White, 1986). Here are some examples. Druart *et al.* (1982) divided the process of adventitious root formation into two phases: "inductive" phase and "initiative" phase, during which root primordia were initiated. De Klerk *et al.* (1995) distinguished three phases in the rooting process of microshoots in apple, i.e. dedifferentiation, induction and morphological differentiation. Mitsuhashi-Kato *et al* (1978a) presented a more detailed schedule which included six successive interdependent phases: induction; transverse first division of pericycle cell(s); longitudinal first division of daughter cells; continued cell division without an increase in gross volume of the meristematic cluster(s); volume increase of cell cluster(s) by cell expansion; and root protruding.

Broadly speaking, it seems that the rooting process could be covered by three stages, namely induction phase (before first cell division), initiation phase (from first cell division to formation of primordial initials), and development phase (from primordial initials organized and formed as primordia to root emergence). Here a

primordium is termed as a group of cells whose structure becomes organized, while a primordial initial refers to a cluster of cells that will further yield a primordium.

During the induction phase, there is little or no anatomical change. The length of this phase varies largely among different species, from several days to several months (Haissig, 1974c). During this period, the potential cells are activated by the appropriate stimulus (e.g. auxin and wound) and begin to divide. The histological locations of the potential cells are variable from species to species (Lovell & White, 1986).

In some plants, potential root primordial initials (or "pre-formed primordia") arise at nodes (e.g. *Salix*) associated with bud gaps (e.g. *Cotoneaster* species, *Ribes*) and leaf gaps (e.g. *Loricera*, *malus* pps.). Others are associated with ray tissue and some authors have noted that the primordia only occur when the rays are abnormally broad, such as in *Juniperinus communis*, var. *depressa*, *J. horizontalis* and *J. virginiana* and a range of other species (see Lovell & White, 1986 and references therein). Typically, at the sites of potential primordial initials of brittle willow (i.e. leaf gaps), the cells develop large nuclei, prominent nucleoli and dense cytoplasm; the number of cells is increased by cell divisions occurring in all planes but with little enlargement of the daughter cells. After excision, these cells further divide and become organized.

In woody plants, experimentally induced primordia may arise either at nodes or at internodes. Occasionally root primordia occur very close to the basal end of a cutting, for example in Norway spruce cuttings (Dalgas, 1973). Root primordia sometimes also develop from external callus which produces at the base of a cutting, e.g. *Aies frima*, *Cedrus liani*, *Cryptomeria japonica* and others (Satoo, 1956), or from internal callus, e.g. *Pseudotsuga menziesii* (Bhella & Roberts, 1975), *Carya illinoensis* (Brutsch *et al.*, 1977), and *Citrus medica* (Carpenter, 1961). In pine cuttings, almost all primordia are initiated within callus tissue (Satoo, 1952, 1955). Lovell & White (1986) pointed out that there are relatively few locations at which primordia arise despite the large number of species. The favoured sites are rays (close to the phloem and cambium) or in bud or leaf gaps.

Lovell & White (1986) concluded that the origin of induced primordia of herbaceous plants is always close to vascular tissue irrespective of the plant species and materials (e.g. stem, hypocotyl, cotyledon, leaf, petiole, etc.). For instance, hypocotyls of *Phaseolus* produce roots in four distinct longitudinal rows parallel to and between the four pairs of vascular bundles. This location of root origin cannot be changed by the application of auxin. However, adventitious roots which developed from epidermis were also reported (Wilson, 1927; McVeigh, 1938).

At the end of induction phase, potential cells begin to respond to the rooting stimuli. It seems that the first histological event of induced primordia is the changes of protoplasm and nuclei. Before the first cell division, protoplasm increased in quantity and density (Hicks, 1987; Rodriguez *et al.*, 1988; Harbage *et al.*, 1993; Jásik & De Klerk, 1997); nuclei enlarge and move to the centres of the cells (De Klerk *et al.*, 1995; Jásik & De Klerk, 1997); and nucleoli become prominent (Harbage *et al.*, 1993). Observation of the ultrastructure of the initial cells in apple stem slices showed an increased proportion of mitochondria, dictyosomes and nuclei at the expense of vacuoles (Jásik & De Klerk, 1997). Following all or some of these changes, the first cell begins to divide.

As a rule, the first division of the potential cells is transversal (Mitsuhashi-Kato *et al.*, 1978a; Jásik & De Klerk, 1997). In some instances, 1 to 3 cells divide first (Carlson, 1929; Naylor & Johnson, 1937) and in others, groups of cells would become root initials.

Root primordia develop by continuous division of the root initials and by incorporation of adjacent cells (Haissig, 1974c). In *Agathis australis*, root initials began to be organized by the time this group of cells was up to about 1500. Periclinal divisions gave rise to three rows of cells and these became the vascular and columella initials (Lovell & White, 1986). Elongation and differentiation of the organized primordia result in the root growing through cortex and emergence.

1.2.2 Mineral elemental aspect

Mineral nutrition is one of many factors that influence adventitious root formation in cuttings. It is likely that all of the mineral elements which are required by plants for

growth in general are essential for root initiation and development as well (Blazich, 1988).

The mobilization of a mineral element into the base of a cutting during root initiation is a strong indication that the nutrient is important at this stage of the root formation process (Blazich, 1988). Moreover, the mobilization of a mineral element depends on the nutrient level of the stock plants from which the cuttings are taken (Blazich, 1988). This means that a mineral element may be redistributed from other regions or parts of the cutting to the region where roots would initiate. However, lack of mobilization should not rule out requirement for a particular nutrient since some, such as Ca, are considered immobile (Mengel & Kirkby, 1982). Calcium was identified as important to root formation in poplar shoots raised *in vitro* (Bellamine *et al.*, 1998). The authors found that rooting was inhibited by 42% if Ca was excluded from the auxin-free medium for root development. Addition of EGTA, a calcium chelator, or lanthanum chloride, a calcium channel blocker, greatly or completely inhibited the rooting process, respectively.

Although adventitious rooting and mineral nutrition are intimately related, the subject is difficult to deal with because root formation is a multi-stage process and few studies have distinguished between effects of minerals at the various stages (Hartmann & Kester, 1983; Blazich, 1988). In addition, the fact that few cells are involved in the rooting initiation in a cutting of some plants makes it more difficult to identify the effects of mineral elements on root formation during the early stage of the process.

The few studies which have examined the mobilization or redistribution of mineral nutrients within cuttings during rooting (mobilization studies) have clearly pointed to the importance of mineral nutrients in root initiation (Blazich, 1988). The results obtained by Stuart (1938) in stem cuttings of kidney bean (*Phaseolus vulgaris* L. cv. Calapproved) and by Strydom & Hartmann (1960) in softwood stem cuttings of plum (*Prunus* L. cv. Marianna 2624) indicated that N was mobilized during root initiation, and the redistribution was accelerated by auxin treatment. However, conflicting reports have been available, for example, N was not mobilized nor was any redistribution of P, K, Ca and Mg detected during root initiation in stem cuttings of Japanese holly (*Ilex crenata* Thunb. cv. Convexa) (Blazich & Wright, 1979; Blazich

et al., 1983). Mobilization of P but not N, K and Ca was reported during root initiation in cuttings of chrysanthemum (*Chrysanthemum morifolium* Ramat. Indianapolis white) (Good & Tukey, 1966). The conflicting reports may somehow be due to differences in plant species (Blazich, 1988).

In apical stem cuttings of poinsettia (*Euphorbia pulcherrima* Willd. Ex. Klotzch), Fe, Cu and Mo accumulated in the basal portions of stem cuttings during early root initiation before root primordia elongation, while P, K, Ca and Mg concentration declined. During root primordia elongation and root emergence, Fe, Cu, Mo, Mg, Mn, B and Zn concentration continued to increase at the cutting bases, but P and K concentrations remained low compared to when cuttings were initially inserted in the propagation medium (Svenson & Davies, 1995).

Electron probe X-ray microanalysis is the most versatile technique for the quantitative estimation of spatial distribution of chemical elements in a biological sample, and offers a special tool for studying the elemental changes during the induction of plant morphogenesis (Pedroso & Pais, 1992) as it allows comparisons of most mineral elements to be made among groups of cells (Pitman *et al.*, 1981). Therefore, it has been adopted to estimate mineral elemental changes and distributions in plant materials by some researchers (Pitman *et al.*, 1981; Drew & Fourcy, 1986; Peterson *et al.*, 1986; Webb & Jackson, 1986; Jaunin *et al.*, 1991; Pedroso & Pais, 1992 1994). The reports on application of this technique to adventitious root formation, however, are very limited.

By using electron probe X-ray microanalysis, Pedroso & Pais (1992) indicated that there was a significant increase of Ca and Fe levels and a decrease of S level during direct and indirect rhizogenesis of *Camellia japonica* leaves.

1.2.3 Physiological and biochemical aspects

1.2.3.1 Physiological aspect

A cutting is in a thermodynamically unfavourable state, since the primary root system which is a part of the physical and physiological support is removed. Therefore, the metabolism in a cutting must have been altered. A higher respiratory rate is expected

after cuttings are taken and induced to root. This is supported by the evidence that the increases in activities of some respiratory enzymes, for instance, succinic dehydrogenase (SDH), cytochrome oxidase (Cox) and NAD-dependent matate dehydrogenase (MDH) were observed during adventitious root formation in cuttings of *Hydrangea macrophylla* (Molnar & LaCroix, 1972b) and *Phaseolus vulgaris* (Upadhyaya *et al.*, 1986).

It has also been suggested that most cuttings with leaves should be rooted in an environment that is not only conducive to photosynthesis (Davis, 1988b), but should also enhance photosynthetic rate in order to synthesize some organic substances for rooting. Photosynthesis could be required to provide carbohydrates, auxin or other substances to the base of the cuttings involved in rooting. Surprisingly, net photosynthesis in pea shoot cuttings decreased rapidly after excision from the stock plant, particularly during the first 24-48 hours (Davis & Potter, 1981). Similar patterns of net photosynthesis have been observed with cuttings of other species and rates were considerably lower than those in intact plants were (Okoro & Grace, 1976; Machida *et al.*, 1977; Eliasson & Brunes, 1980). The decrease of net photosynthesis is presumably due to stomata closure after excision of the cuttings of *Cornus* and *Rhododendron* (Gay & Loach, 1977) and of aspen (*Populus tremula* × *P. tremuloides*) and of willow (*Salix caprea* × *S. viminalis*) (Eliasson & Brunes, 1980). However, dry matter accumulation significantly increased in the cuttings during the rooting process despite of low rates of net photosynthesis. Net photosynthesis continued at relatively low rates till root emergence and then gradually increased thereafter. The increase was probably at least in part due to the alleviation of water stress and re-opening of stomata (Davis, 1988b). Therefore, the success in rooting cuttings depends to a considerable extent on maintaining a satisfactory water balance in the tissues by different treatments (Loach, 1988b).

Water stress can also directly influence net photosynthesis via non-stomata effects (Farquhar & Sharkey, 1982). It is not very clear how these changes are related to root formation, but they may be important in generating the metabolic energy required for the rooting process (Upadhyaya *et al.*, 1986). By using uncouplers or inhibitors of oxidative phosphorylation, some researchers (Turetskaya & Kof, 1965;

Krul, 1968; Nanda *et al.*, 1978) showed that oxygen was required during the rooting process.

1.2.3.2 Endogenous phytohormones

Auxin It is well-known that auxin has a central role in the initiation and development of adventitious roots (Jarvis, 1986). However, auxin has not been considered as the sole determinant for adventitious root formation (Gurumurti *et al.*, 1984; Haissig, 1986; Jarvis, 1986; Gaspar & Coumans, 1987; Gaspar & Hofinger, 1988).

Gaspar & Hofinger (1988) stated that "several strong themes, however, can be gleaned from the literature: 1) there is a good positive correlation between the endogenous free auxin content and the percent of rooting when auxin level is high at the time cuttings are made; 2) free auxin increases in the rooting zone prior to rooting; and 3) the rapid decrease in free IAA level immediately preceding root initiation coincides with the formation of IAA conjugates."

A high level of endogenous auxin is required during the root-inducing stage (Bláhová, 1969; Michniewicz & Kriesel, 1970), while a reduced level of endogenous auxin has been implicated in the failure of rooting in a number of species of plant cuttings (Smith & Wareing, 1972a 1972b). The investigation conducted by Brunner (1978) demonstrated that the levels of endogenous IAA and IBA at the rooting region of control and auxin treated hypocotyls in *Phaseolus vulgaris* L. increased markedly during the first 24 hours after excision of the hypocotyls. The IAA and IBA concentrations decreased thereafter and root primordia were observed between 48 and 96 hours after the excision of hypocotyls.

Baraldi *et al.* (1995) also showed that a dramatically early increase followed by a decrease of free IAA was seen in microcuttings of pear 'Conference', an easy-to-root cultivar. Meanwhile, a significant increase of IAA conjugate and free putresine was observed in 'Doyenne d' Hiver', a difficult-to-root cultivar. In apple microcuttings, following treatment with IBA endogenous levels of free IAA increased markedly during the first 3 hours and subsequently decreased slowly in both easy-to-root and difficult-to-root microcuttings (Noiton *et al.*, 1992). Research on plants in

waterlogged soils showed an accumulation of auxin at the stem base (Philips, 1964; Wample & Reid, 1979), which subsequently formed adventitious roots.

Based on application of radioactive labelled auxins and several techniques such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC), some researchers (Epstein & Lavees, 1984; Alvarez *et al.*, 1989; Baraldi *et al.*, 1995; Visser *et al.*, 1995) have indicated that the increase of free IAA concentration at rooting region was of the conversion of exogenously applied auxins or transport of endogenous IAA from shoots. Whereas the decrease of endogenous IAA concentration could be due to degradation either chemically by factors such as acidic pH, heat, light, oxidants, peroxides or heavy metals, or enzymatically by certain metalloproteins such as peroxidase and/or IAA-oxidase (Jarvis, 1986; Bhattacharya, 1988; Gaspar & Hofinger, 1988). In addition, the decrease in endogenous levels of free IAA may be caused by its conjugation with other substances such as amino acids, sugars and inositol. For example, Collet & Le (1987) showed that a brief pretreatment of the cut ends of rose and apple shoots with a high concentration of IAA produced best rooting. In this experiment, there was first an accumulation of IAA in the root zone, followed by the rapid disappearance of IAA and the appearance of IAA conjugates. The conjugation is a reversible inactivation as the free auxin may be released from the conjugates (Smulders *et al.*, 1990; De Klerk *et al.*, 1999).

There are some cases in which auxin levels did not seem to be associated with root initiation and development. For instance, Stoltz (1968) found that endogenous auxin concentration in *Chrysanthemum* spp. was not related to rooting. And Böttger (1978) did not detect a change in the level of IAA although the number of lateral root primordia arising from root of *Pisum sativum* increased significantly. In these studies, the possibility that more advanced or sensitive techniques for IAA determination might lead to a different conclusion cannot be ruled out.

Ethylene Ethylene is another hormone that has often been associated with adventitious root formation (Robbins *et al.*, 1983 1985; Jusaitis, 1986; Riov & Yang, 1989; Visser *et al.*, 1996). However, a preponderance of evidence suggests that endogenous ethylene is not directly involved in auxin-induced rooting of cuttings or

flood induced rooting of intact plants (Batten & Mullins, 1978; Mudge & Swanson, 1978; Geneve & Heuser, 1983; Mudge, 1988; Geneve *et al.*, 1990; Harbage & Stimart, 1996b). For instance, Harbage & Stimart (1996b) found that apple microcuttings of easy-to-root cultivar 'Gala' and difficult-to-root cultivar 'Triple Red Delicious' differed significantly for root count but not for ethylene evolution when treated with IBA. Ethylene production was stimulated by auxin but was not related to adventitious root formation.

Abscisic acid (ABA) Only a few investigations have measured endogenous ABA content in relation to adventitious root formation, although ABA content can be determined relatively easily, especially compared to the measurement of other plant growth substances (Davis, 1988a). In general, ABA is considered to oppose the action of gibberellins which normally inhibit adventitious root formation (Davis, 1988a) and to be a "rooting cofactor" (Chin *et al.*, 1969). Wu *et al.* (1981) reported that *Rhododendron* pps. cuttings rooted best at times of the year when endogenous ABA concentrations in the stems were highest and when the contents of no other endogenous hormones were correlated with rooting capacity, suggesting that ABA may play an important role in regulating rooting of *Rhododendron* cuttings. Blake & Atkinson (1986) obtained similar findings. However, in some plant species endogenous ABA appears to be associated with inhibition of root formation (Maldivey *et al.*, 1986; Pelese *et al.*, 1989; Noiton *et al.*, 1992).

Cytokinins It is now generally accepted that cytokinins are synthesized in roots and transported to the above-ground portions of the plant via the xylem (Letham & Palni, 1983; van Staden & Harty, 1988). Kriesel (1976) found that the cytokinin levels in the shoots of *Salix* pps. which contained potential primordial initials (preformed primordia) increased prior to visible emergence of adventitious roots. This means that cytokinins are produced by root primordia. The results of Featonby-Smith & van Staden (1981) also indicate that cytokinin synthesis and transport begin before the new adventitious roots rupture the petiole epidermis of *Phaseolus vulgaris* leaf cuttings, which in this case occurred at day 6 after the cuttings were taken.

This seems to be contrary to the generally accepted consideration that cytokinins are inhibitors of adventitious root formation, although this warrants a closer analysis.

For example, Bridglall & van Staden (1984) found that exogenous application of auxin to leaf cuttings of *Phaseolus vulgaris* first resulted in an increase in cytokinin content, and then there was a redistribution of cytokinins within the cuttings. The net effect was apparently to decrease cytokinin activity to a level where it was either promoting or no longer inhibitory to adventitious root formation.

Gibberellins Of the more than 70 different naturally occurring gibberellins (Sponsel, 1985), GA_3 is the only gibberellin that has been extensively studied in adventitious root formation (Hansen, 1988). Adventitious root formation, which is stimulated by auxin, is inhibited by GA_3 (Hansen, 1988).

Few investigations concerning changes in content of endogenous gibberellins during rooting have been made (Hansen, 1988). Bláhová (1969) reported that endogenous gibberellin activity in the basal part of the cuttings from epicotyls of pea (*Pisum sativum* L.) decreased during the first 24 hours after cuttings were taken and then increased gradually over the next 48 hours. In tissue cultures of apple (*Malus pumila* Mill. cv. Jonathan), Takeno *et al.* (1982/83) found that the endogenous gibberellin-like substances decreased with increasing number of subcultures while the percentage of rooted cuttings increased. This increase in rooting percentage could be due to changes in factors other than gibberellin content but the correlation is worth noting (Hansen, 1988).

1.2.3.3 Enzyme activity

Many enzymes, including oxidases, hydrolytic enzymes, respiratory enzymes, have been determined during adventitious root formation in various plant species. The changes in activities of a number of enzymes during rooting lead to the suggestion that some of these enzymes may be involved in this process (Chandra *et al.*, 1971; Nanda *et al.*, 1973; Bhattacharya *et al.*, 1976a 1978; Bassuk *et al.*, 1981; Haissig, 1982a; Upadhyaya *et al.*, 1986; González *et al.*, 1991; Gaspar *et al.*, 1992; García-Gómez *et al.*, 1995; De Klerk, 1996; Pan & Tian, 1999). In particular, peroxidase, polyphenol oxidase, IAA-oxidase and amylase have often been studied in relation to adventitious root formation.

Peroxidase (PO) As described previously, it is generally accepted that the primary events of root formation, i.e. dedifferentiation and formation of a meristematic locus, are associated with a peak in endogenous IAA levels. Meanwhile, there is a peak of PO activity following that of IAA level prior to root formation (Berthon *et al.*, 1989; Moncousin *et al.*, 1989; De Klerk, 1996). The inverse relationship between the endogenous IAA level and PO activity suggests that the basic POs may be involved in the oxidation of IAA (De Klerk, 1996).

Many studies have attempted to correlate PO activity with rooting. Pan & Tian (1999) suggested that PO was positively correlated with rooting in mung bean hypocotyl cuttings as the PO activity in the rooting zone coincided with root development. Rout *et al.* (2000) also reported that PO activity increased considerably during root induction of microshoots of *Psoralea corylifolia in vitro.* A two-step system of PO response to wounding or stress proposed by Gaspar *et al.* (1985a) showed that the rapid increase in basic PO activity was associated with auxin metabolism and led to the induction of rooting, whereas acidic PO activity was linked to lignification during root initiation and development. Hagége *et al.* (1988) also reported that PO plays an important role in lignin biosynthesis that is involved in root primordium initiation and development. Jarvis (1986), Gaspar *et al.* (1990) and Moncousin *et al.* (1988) reported that the PO peaks were associated with the first cell division, i.e. the PO peak terminated the inductive or initiative phase of root formation. The studies on isoenzyme polymorphism showed that PO isoenzymes were positively correlated with initiation and development of rooting in several plant species (Dhaliwal *et al.*, 1974; Bhattacharya *et al.*, 1978; Thorpe *et al.*, 1978; Moncousin & Gaspar, 1983).

Histological staining allows localization of enzyme activity directly in plant tissues and monitoring of the changes of enzyme activities in different tissues during rooting. For example, Molnar & LaCroix (1972b) reported that PO was the first enzyme which increased in activity at the sites of root primordial initiation during the rooting process in *Hydrangea macrophylla* stem cuttings. González *et al.* (1991) also observed PO activity in differentiating xylem cells and root primordia in hazelnut (*Corylus avellana* L.) cotyledon cuttings. By application of antibody, García-Gómez

et al. (1995) found that during the process of root formation in avocado (*Persea americana* M.) microcuttings PO activity was closely associated with the growth and differentiation process and also with the developing root primordia.

Based on the foregoing results, PO has been considered as a useful biochemical marker to predict the success of vegetative propagation *in vitro* and *in vivo* (Thorpe *et al.*, 1978; Gaspar *et al.*, 1985a 1992; Hand, 1994; García-Gómez *et al.*, 1995; De Klerk, 1996).

However, some researchers detected neither the peak nor subsequent decrease in PO activity (Gebhardt, 1985; Patience & Alderson, 1987; Pythoud & Buchala, 1989; De Klerk *et al.*, 1990) or any particular isoenzymes during rooting (García-Gómez *et al.*, 1995). Gaspar *et al.* (1992) considered that this may be due to the fact that in these studies, PO activity was measured in purified extracts and/or not expressed per unit protein.

IAA-oxidase (IAA-O) Like PO, IAA-O has also been considered as one of the enzymes which are associated with metabolism of endogenous IAA or regulation of the concentration of auxin in cuttings during root formation (Bhattacharya, 1988). Extensive studies on IAA-O activity and rooting were carried out in a number of plant species by Nanda and associates (Dhaliwal *et al.*, 1974; Nanda *et al.*, 1974; Bhattacharya *et al.*, 1978; Chibbar *et al.*, 1979; Bansal & Nanda, 1981; Pan & Gui, 1997). Dhaliwal *et al.* (1974) found that a high IAA-O activity was associated with rooting in a medium containing different concentrations of glucose and IAA. Bhattacharya *et al.* (1977) also reported that an increased IAA-O activity during the first 20-40 hours was associated with increased rooting in hypocotyl cuttings of *Phaseolus mungo*. Investigation conducted by Bansal & Nanda (1981) in the cuttings of easy- and difficult-to-root species, including *Salix tetrasperma*, *Populus robusta* L., *Hibiscus rosa-sinensis* L., and *Eucalyptus citriodora* Hook which rooted 100%, 100%, 30% and 0%, respectively, showed that IAA-O activity was highest in those cuttings where rooting was most pronounced and least in those with few to no roots. IAA-O activity was also associated with rooting in some other plant species (Pingel, 1976; Brunner, 1978; Chibbar *et al.*, 1979; Pan & Tian, 1999).

By comparing isoenzyme profiles of IAA-O and PO in cycloheximide treated hypocotyl cuttings of *Impatiens balsamina*, it was demonstrated that some isoenzymes of IAA-O corresponded with isoenzyme profiles of PO (Dhaliwal *et al.*, 1974). An analogous isozymic reinforcement could also be observed in PO zymogram and IAA-O zymogram of the extracts from hypocotyl cuttings of mung bean (*Phaseolus aureus* Roxb.) (Frenkel & Hess, 1974). Nanda *et al.* (1975) postulated that IAA-O consists of two active sites: one representing the oxidase (Site I) which was concerned with production of active IAA oxidation products, the other (Site II) merely detoxifying the supraoptimal concentration of IAA for rooting. Therefore, some POs seem to act as IAA-Os. Pan & Tian (1999) thought that IAA-O, like PO, was also positively correlated with rooting in mung bean hypocotyl cuttings. But PO and IAA-O activities in all treatments started 24 hours and 12 hours after cutting, respectively, leading to the suggestion that the major role of IAA-O differed from that of PO in adventitious root formation.

However, contrasting viewpoints exist. For example, Haissig (1974a) suggested that the available evidence (Stenlid, 1963; Tomaszewski & Thimann, 1966) seems to refute the IAA oxidation-growth control theory both in general and in the specific case of root primordial initiation.

Polyphenol oxidase (PPO) Despite years of research, the physiological role of PPO is not clear (Mayer, 1987; Vaughn *et al.*, 1988). It was reported that PPO is able to catalyze the hydroxylation of phenolic compounds *in vitro*, but it is not involved in synthesis of phenolic compounds in intact cells *in vivo* by using specific inhibitors (Duke & Vaughn, 1982; Strack *et al.*, 1986). It has been shown that applied phenolics could enhance rooting (Hess, 1969; Nanda, 1975; Bassuk *et al.*, 1981). This has led to the suggestion that PPO may oxidize endogenous or applied phenolics which then conjugate with oxidation products of auxin to form cofactors necessary for rooting (Bhattacharya, 1988). However, when cuttings are made cell damage by wounding allows enzyme and substrates to come into contact, thereby facilitating the oxidation of phenolics (Hand, 1994).

Positive correlation between PPO activity and rooting has been reported in *Pistacia vera* (Al Barazi & Schwabe, 1984), *Salix tetrasperma* (Bhattacharya *et al.*,

1978), *Populus nigra* (Bhattacharya *et al.*, 1976b), apple (Bassuk *et al.*, 1981), *Phaseolus vulgaris* (Upadhyaya *et al.*, 1986), *Corylus avellana* (hazelnut) (González *et al.*, 1991), *Phaseolus aureus* (Frenkel & Hess, 1974). Histological investigation indicated that increased PPO activity was localized at the sites where roots initiated (Smith & Thorpe, 1975a; Al Barazi & Schwabe, 1984) and in root primordia (González *et al.*, 1991). Electrophoretic studies showed that some new PPO isoenzymes appeared during rooting, suggesting that these enzymes were associated with rooting (Frenkel & Hess, 1974; Bhattacharya *et al.*, 1976b 1978). It is worthwhile to point out that some isoenzymes showed a multiple enzymatic function in PO, IAA-O and PPO. This is supported by the finding of Frenkel & Hess (1974), in which an electrophoretic band showing an intensification during root initiation had an identical Rf value in all cases of PO, PPO and IAA-O staining. However, in mung bean (*Vigna radiata* L. Wilczek) seedlings Duke & Vaughn (1982) demonstrated that PPO is not involved in metabolism of phenolic compounds in developing plant tissues.

Amylase (Amy) A close relationship between disappearance of starch and increase of Amy activity during rooting was demonstrated in cuttings of *Bryophyllum tubiflorum* (Nanda *et al.*, 1967), *Populus nigra* (Nanda *et al.*, 1969; Nanda & Anand, 1970), *Hydrangea macrophylla* (Molnar & LaCroix, 1972b), and *Malus domestica* (apple) (Jásik & De Klerk, 1997). Borthwick *et al.* (1937) showed that a high level of carbohydrates during root initiation was needed. Therefore, a high Amy activity during rooting might be expected in starch storing plant species for a sugar supply (Bhattacharya, 1988). In *Populus nigra*, Nanda & Anand (1970) demonstrated the relationship between rooting and seasonal fluctuation of Amy activity. Amy activity was high during the season (April-August) when rooting was profuse, but it decreased during the season (October) when rooting decreased and it was not detected in December when rooting was poor.

By using a modified method for animal tissues, Molnar & LaCroix (1972b) histologically investigated Amy activity in *Hydrangea macrophylla* cuttings during root formation. Highest Amy activity was demonstrated in the epidermal tissues and vascular bundles at the time when cuttings were made. As the root primordia developed, very high Amy activity was revealed behind and around the primordia and

starch disappeared in this area. The foregoing finding suggested that hydrolysis of starch by Amy provided sugars as energy for adventitious root formation.

Similar to PO, PPO and IAA-O, new isoenzymes of α and β Amy were also observed in etiolated segments of *Populus nigra* cultured in glucose plus IAA where rooting was profuse but these isoenzymes did not appear in the presence of inhibitors of nucleic acid and protein synthesis which also initiated root formation, suggesting that these enzymes including Amy was associated with rooting (Bhattacharya *et al.*, 1976b).

Upadhyaya *et al.* (1986) also found that Amy activity increased dramatically immediately following excision, but they considered that the increase was probably due to a wounding response and Amy was not directly related to rooting because the treatment with paclobutrazol which enhanced rooting did not promote Amy activity.

Invertase (INV) Bhattacharya & Nanda (1978) investigated the changes in INV activity and its relation with anatomical differentiation of root primordia in cuttings of *Phaseolus mungo* treated with purine and pyrimidine bases singly as well as in combination with sucrose plus IAA. These treatments enhanced rooting in the cuttings and two distinct peaks in INV activity were observed. The authors suggested that the upsurge of INV activity prior to rooting seemed to "balance" the level of soluble sugars with auxin level and thereby promote rooting.

Phosphatases The development of root primordia may dispose of tissues which block their path to the outside by either crushing or hydrolysis (Haissig, 1974c). This process could be completed by both enzymatic hydrolysis (by pectinase, protease, nuclease, etc.) and mechanical destruction and associated bioenergetic suitable for overcoming the constraints of physical barriers to emerging roots (Bhattacharya, 1988). Phosphatases have been hypothesized to be involved in this bioenergetic process and were measured in the cuttings of *Phaseolus mungo* during rooting by Bhattacharya & Nanda (1979). Their results showed that phosphatases remained low at the early stage of rooting and increased subsequently, which suggested increased energy requirement was associated with the emergence of roots. This postulate was supported by the researches on the relationship between rooting and application of

uncouplers of oxidative phosphorylation (Nanda *et al.*, 1978), and cobalt and sodium azide which counteract the uncouplers (Nanda & Dhawan, 1976).

Respiratory enzymes By using uncouplers or inhibitors of oxidative phosphorylation, some researchers (Turetskaya & Kof, 1965; Krul, 1968; Nanda *et al.*, 1978) adequately demonstrated the requirement for oxygen during rooting. Molnar & LaCroix (1972b) histologically examined succinic dehydrogenase (SDH) and cytochrome oxidase (Cox) in the cuttings of *Hydrangea macrophylla* during rooting. SDH activity was very low when the cutting were made and it could be observed in the root initial area only at day 3 after the cuttings were made. Cox was found to increase in activity and could be detected in the root initials 2 days after the cuttings were made. Both SDH and Cox activities were further increased with the development of root primordia. Upadhyaya *et al.* (1986) found that NAD-dependent malate dehydrogenase (NAD-dependent MDH) was dramatically increased by paclobutrazol, which enhanced rooting of hypocotyl cuttings in *Phaseolus vulgaris*, suggesting that NAD-dependent MDH activity was correlated with rooting.

1.2.3.4 Carbohydrates

The early investigation on the relationship between carbohydrates and root formation was performed by Kraus & Kraybill (1918). Their results led to the widely accepted hypothesis that high C/N ratios in cuttings are conducive to the root formation whereas the low C/N ratios reduce rooting capacity.

Accumulation of sugars and starch in cuttings was firstly found in leaves, then in the stems and subsequently in the base of cuttings in *Pisum sativus* (Veierskov & Andersen, 1982) and in *Pinus banksiana* (Haissig, 1982b). These findings clearly indicate that the carbohydrates were synthesized in leaves and transported to the base of the cuttings where roots formed. In cuttings with leaves, a decrease of carbohydrate content may be observed during first few days after the cuttings were made (Haissig, 1982b; Veierskov *et al.*, 1982). This decrease may be due to the closure of stomata which diminishes the cutting's ability to fix CO_2 (Veierskov, 1988). However, a net accumulation of carbohydrates normally occurs until roots emerge from leafy cuttings under light condition (Lovell *et al.*, 1974; Hansen *et al.*, 1978;

Davis & Potter, 1981; Veierskov *et al.*, 1982). Rapid accumulation of carbohydrates has also been observed in cuttings if auxin or increased rooting temperature (15-25°C) were applied to hasten rooting (Altman & Wareing, 1975; Haissig, 1982b; Veierskov & Andersen, 1982)

The research on this subject during the past 80 years has demonstrated that there can be a relationship between carbohydrates and root formation in cuttings, although contrasting reports occasionally arise (see review by Haissig, 1986; Veierskov, 1988). The controversy is probably due to the carbohydrate status of the stock plant and cuttings which is determined by environmental and developmental parameters and other factors known to influence rooting. Hence the influence of carbohydrates on rooting may often be masked by other overriding factors (e.g. auxin, state of differentiation) (Veierskov, 1988). In addition, carbohydrate content has primarily been determined from whole tissue homogenates and this may mask what is happening in primordial cells, particularly in the cases where only a few cells are involved in the root primordial initiation. Therefore, determination of carbohydrates at the histological level would greatly enhance our understanding of the relationship of carbohydrates to rooting.

For example, Molnar & LaCroix (1972b) found that there was a positive correlation between the number of roots initiated and the starch content of the cuttings and that starch disappeared from the endodermis, phloem and xylem rays, and pith in close proximity to the developing root primordia. This disappearance of starch in specific areas was associated with increased amylase activity as mentioned previously. Recently, Jásik & De Klerk (1997) demonstrated that large numbers of starch grains appeared in vascular bundles close to the basal part of the stem slices of apple (in parenchymatous cells of xylem and phloem and immature tracheal and phloem elements) after 24 hours of IBA treatment (rooting), whereas starch grains appeared later and in lower numbers in control treatment. After 2 days, the proportion of starch grains decreased appreciably. The authors suggested that the accumulation of starch grains during the first day functioned as a transient storage for carbohydrates before cells entered division. A similar rapid hydrolysis of starch was found during adventitious root formation in grapevine shoots (Fabbri *et al.*, 1985). The findings suggest that starch may play an important role in adventitious root formation.

1.2.3.5 Polyamines

Polyamines have been shown to play a role in a variety of biological process in a wide range of organisms (Galston & Kaur-Sawhney, 1987; Hand, 1994). Jarvis *et al.* (1983b) demonstrated that applications of exogenous polyamines could enhance rooting of hypocotyl cuttings in mung bean. Nevertheless, in a review of the role of polyamines in adventitious root formation, Sankhla & Upadhyaya (1988) pointed out that there was limited evidence to link polyamines to the regulation of rooting although some studies had shown the effect of polyamines on rooting. However, the results of later studies have found a correlation between increased polyamine levels with cell division and root primordial development (Tiburcio *et al.*, 1989; Biondi *et al.*, 1990; Geneve & Kester, 1991), suggesting changes in polyamines could be useful markers of the rooting process (Hand, 1994).

1.2.3.6 Phenolics (Flavonoids)

In his review of phenolic metabolism during rooting, Haissig (1986) pointed out that anabolism of simple and complex phenolics should be important in the rooting process as aromatic amino acids are involved in the synthesis of proteins and phenylpropanoids linked with lignin biosynthesis. Investigations on the relationship between endogenous phenolic level and rooting are very limited, compared with other biochemical substances, e.g. auxin, some enzymes, and carbohydrates.

Druart *et al.* (1982) demonstrated that the levels of endogenous phenolics increased in the shoot cuttings of apple during rooting, which is consistent with observation that phenolics accumulate as a result of wounding (Lewis, 1980). In *Pinus banksiana* (jack pine) seedling cuttings, Montain *et al.* (1983a, 1983b) found a significant basipetal movement of soluble phenolics during propagation and before rooting. The phenolics were synthesized in the terminals, i.e. buds plus some subtending needles, which were transported basipetally to the base of the cuttings. The accumulation of the phenolics at the base of the cuttings may be involved in lignification of tracheid nests that formed in the callus tissue where root primordia initiated. As described previously, phenolics may be oxidized mainly by PPO, leading to the formation of rooting cofactors by inhibiting IAA oxidase activity (Haissig,

1974a). This should result in high levels of endogenous IAA which in turn would stimulate rooting (Curir *et al.*, 1990). PO may also catalyze the formation of quinones from phenolics, thus mimicking catalysis by PPO (Vaughn & Duke, 1984). Quinones are required in the biochemical pathway of formation of auxin-phenolic conjugates that could favour rooting. However, some studies suggested that low endogenous levels of simple phenolics in stock plants favour rooting of cuttings (Fadl *et al.*, 1979; Druart *et al.*, 1982).

1.2.4 Molecular aspects

During Auxin-induced root formation either in epicotyl or hypocotyl and other plant tissues, increased synthesis or appearance of new rooting-specific DNA, RNA and proteins have been reported (Kantharaj *et al.*, 1979; Dhindsa *et al.*, 1987; Kantharaj & Padmanabhan, 1990; Macisaac & Sawhney, 1990; Oliver *et al.*, 1994; Kang, 1995). This is perhaps not surprising, given that the process involves cell division and major changes in the metabolism of the cells which redifferentiate to form root primordia (Hand, 1994).

As described previously, activities of specific enzymes have been shown to vary by general gross assay methods, histochemical staining and isoenzyme identification. Protein changes during rooting have also been studied by total protein, histochemical staining and electrophoresis.

Auxin-induced adventitious root formation has been associated with an increase of general soluble proteins (Kantharaj *et al.*, 1979) and some specific proteins (Dhindsa *et al.*, 1987). In addition to wound responses and other events (Oliver *et al.*, 1994), the increase of the proteins may be due to the alteration of gene expression during the process (Dhindsa *et al.*, 1987; Hand, 1994; Kang, 1995).

In the hypocotyl cuttings of *Phaseolus vulgaris*, Kantharaj & Padmanabhan (1990) found that both IBA and BA independently and in combination enhanced the rate of protein synthesis very early (30 minutes after treatment) without augmenting or activating transcriptional activity. In addition, cycloheximide could inhibit early protein synthesis and delay root initiation. The authors suggested that the early enhancement (30 minutes) in the rate of protein synthesis was due to hormonal

activation of protein synthesizing machinery and not due to early synthesis of total RNA including mRNA, and that the increased protein synthesis at later stages was augmented by the enhanced RNA including mRNA synthesis.

By using microautoradiography, Tripepi *et al.* (1983) demonstrated that there was an 8-11 hour lag period prior to active [^{3}H]-thymidine incorporation into rooting-zone parenchyma nuclei of hypocotyl cuttings of *Vigna radiata*, suggesting that many nuclei were in G_1 phase of the cell cycle when the cutting was taken. Thus the rooting-zone parenchyma cells had entered the S phase of the cell cycle and [^{3}H]-thymidine was incorporated into DNA at 11 hours after the cuttings were placed in the labeling solution. Meanwhile, [^{3}H]-uridine was incorporated into the RNA of the rooting-zone parenchyma cells at 2 hours after the cuttings were placed in the labeling solution. Based on Setterfield's wound-healing study (1978), the authors suggested that the [^{3}H]-uridine in the nucleoli in 2 hours was incorporated into mRNA and that most of the [^{3}H]-uridine observed in the cytoplasm of the rooting-zone parenchyma cells from 8-26 hours was probably in the form of ribosomal RNA.

Auxin-treated specific proteins, which were supposedly associated with rooting, have been detected in the extracts of some plant species during adventitious root formation using the techniques of SDS-PAGE and 2D-PAGE, (Dhindsa *et al.*, 1987; Kantharaj & Padmanabhan, 1990; Macisaac & Sawhney, 1990; Oliver *et al.*, 1994; Kang, 1995). Macisaac & Sawhney (1990) found that NAA-stimulated lateral root formation of *Lactuca sativa* was associated with an increase in soluble proteins as well as the synthesis of several polypeptides, including specific polypeptides with apparent molecular weight of 32 and 31 kDa. The synthesis of these polypeptides coincided with the onset of cell division in pericycle of the NAA-treated roots. Dhindsa *et al.* (1987) reported the changes in the pattern of protein synthesis and in the translatable mRNA production during adventitious root formation of *Vigna radiata*. After 2D-PAGE, they found that several proteins, predominantly of low molecular weights and high pIs accumulated specifically in auxin-treated cuttings. These auxin-induced proteins appeared between 6 and 12 hours after auxin treatment, reaching a maximum at 24 hours and then beginning to decline at 48 hours. Oliver *et al.* (1994) investigated the alteration of gene expression at the level of protein

synthesis and transcript abundance associated with the early events occurring in the de-rooted hypocotyls of sunflower (*Helianthus annuus*). In this study, 7 new proteins could be tentatively ascribed to the process of adventitious root formation and phloem fiber deposition. Using 1D SDS-PAGE, Kang *et al.* (1995) did not detect changes in protein pattern during root formation from gladiolus callus.

Isolation and identification of genes expressed during morphogenesis, such as embryogenesis in carrot cell suspensions (Aleith & Richter, 1990), floral initiation in tobacco (Meeks-Wagner *et al.*, 1989; Kelly *et al.*, 1990) and *Sinapis alba* (Melzer *et al.*, 1990), and adventitious root formation in mungbean (Chen *et al.*, 1996), loblolly pine (Hutchison *et al.*, 1999) and apple (Butler & Gallagher, 1999), have recently been attempted via cDNA cloning. Interestingly, Chen *et al.* (1996) have identified and characterized two cDNA clones (*MII-3* and *MII-4*) corresponding to auxin-induced mRNA by differentially screening a λgt 11 cDNA library constructed from mungbean (*Vigna radiata*) hypocotyls. These two cDNAs are encoded by different genes and were highly expressed in IAA treated hypocotyls, but there was no expression in control and IAA treated leaves. Using 5'- and 3'-rapid amplification of cDNA end cloning, Hutchison *et al.* (1999) found a gene that was highly similar to the α-expansin gene family in angiosperms and expressed during adventitious root formation in hypocotyls of loblolly pine. Butler & Gallagher (1999) isolated and characterized a cDNA clone encoding a novel 2-oxoacid-dependent dioxygenase which is up-related during adventitious root formation in apple stem discs.

1.3 Exogenous factors affecting adventitious root formation

1.3.1 Auxin and other plant growth substances

The changes in the levels of some endogenous plant hormones, which are associated with adventitious root formation, were reviewed previously in Section 1.2.3.2. The effects of applied plant growth substances on adventitious rooting, particularly auxins will be focused on in this section.

1.3.1.1 Auxins

As described previously, it is widely accepted that auxin plays a central role in the initiation and development of adventitious roots (Haissig, 1972 1974a; Haissig, 1986; Jarvis, 1986; Gaspar & Hofinger, 1988; Blakesley *et al.*, 1991). Exogenous application of naturally occurring auxin like IAA and synthetic auxins such as IBA, NAA and 2,4-D is able to overcome the inhibition of root formation or shorten the time to the onset of adventitious root development in many plant species (Jarvis & Shaheed, 1986; Visser *et al.*, 1995). The importance of exogenous auxins can also be indicated by using antiauxins. Application of the antiauxins, PCIB and PBA, to poplar shoots cultured *in vitro* completely inhibited NAA-induced rooting (Bellamine *et al.*, 1998).

Non-woody stem cuttings are usually highly responsive to supplied auxins, although the response is dependent upon the age of stock plant, the auxin itself and its concentration, the duration of the treatment and the time interval between the excision of the cuttings and commencement of auxin treatment (Jarvis, 1986). One of the most commonly used plant growth substances is IBA (Jackson & Harney, 1970; Davis, 1986; van der Krieken *et al.*, 1992), which has only weak auxin activity but is relatively stable, effective and not readily translocated (Gianfagna, 1987). Fernqvist (1966) found that the cuttings from light-grown seedlings of *Phaseolus vulgaris* were most responsive to IBA, then to NAA and least to IAA at the same concentration for a period of 24-hour treatment. Recently, Pan & Tian (1999) found that root number in mung bean hypocotyl cuttings were significantly increased by the treatments with 5,6-Cl-2-IAA-Me (5,6-dichloroindole-3-acetic acid methyl ester) and BSAA (3-(benzo-β-selenienyl) acetic acid) at lower concentrations, compared with the IBA treatment.

Below the phytotoxic level, the higher concentrations of auxin usually result in the more roots formed per cutting. The age of cutting, the time interval from excision to rooting treatment, is negatively related to rooting. For example, Jarvis *et al.* (1983a) demonstrated that the number of roots per cutting decreased with the increasing age of the cuttings from light-grown seedlings of *Phaseolus aureus*. Duration of auxin treatment is another factor that influence rooting. Kantharaj *et al.* (1979) investigated the relationship between IBA treatment intervals and rooting of

Phaseolus vulgaris cuttings. The number of roots per cutting increased dramatically when cuttings were treated with Hoagland's solution containing 10 μg/ml of IBA within 10 minutes, but the number of roots per cutting then declined a little when the cuttings were treated with the solution for 10 to 30 minutes.

It seems that the applied auxins might exert their action through their conversion into IAA. Dunberg *et al.* (1981) found that the IAA level at the bases of IBA-treated cuttings of *Pinus sylvestris* was three times higher than that in the untreated cuttings. Meantime, they did not find any differences in IAA metabolism or transport between the treated and untreated cutting, leading to the conclusion that applied IBA was converted to IAA by the cuttings. This conclusion has been confirmed by later studies on *Olea europaea* and *Vitis vinifera* (Epstein & Lavee, 1984) and *Persea americana* (García-Gómez *et al.*, 1994).

1.3.1.2 Plant growth substances other than auxins

Although auxin appears to be crucially important to adventitious rooting, many of the other presently known plant growth substances have also been shown to either stimulate or inhibit rooting of cuttings (Smith & Thorpe, 1975b; Fabijan *et al.*, 1981; Selby *et al.*, 1992; Visser *et al.*, 1995).

Ethylene Ever since it was demonstrated that application of high concentrations of auxin will stimulate ethylene production, many researchers have investigated the relative roles of auxin and ethylene in the process of auxin-stimulated adventitious root formation by cuttings (Reid, 1987). The studies, including the effects of ethylene, ethylene production, and inhibitors of ethylene synthesis, result in conflicting interpretations of role of ethylene in rooting. In a review of the effect of ethylene on rooting, Mudge (1988) summarized the results of rooting experiments using ethylene or ethephon with a total of 52 plant species, across a wide range of experimental conditions and taxonomic groups. Sixty-eight percent of the experiments demonstrated that ethylene or ethephon stimulated rooting. If these plants are divided into herbaceous and woody plants, 73% of herbaceous plants and 56% woody plants positively responded to ethylene, respectively. This comparison suggests that the degree of tissue lignification might be a factor in the response to

ethylene. Mudge (1988) concluded that the effect of ethylene on adventitious root formation is highly variable depending upon plant species, the environmental and physiological conditions. Furthermore, the promotion of rooting by ethylene is more frequent in intact plants, cuttings of herbaceous plants and plants with preformed root initials than in cuttings of woody plants and plants without preformed root initials, and that the conflicting results may be due to the responsiveness of plant tissues to ethylene over time.

Abscisic acid (ABA) In a number of studies, exogenous application of ABA has been found to stimulate adventitious root formation (Rajagopal *et al.*, 1971; Hartung *et al.*, 1980; Rasmussen & Andersen, 1980; Tari & Nagy, 1996). However, the application of ABA has also been reported to inhibit rooting (Heide, 1968; Krishnamoorthy, 1972; Venverloo, 1976) or to have no effect on rooting (Krelle & Libbert, 1969; Read & Hoysler, 1971; Biran & Halevy, 1973a). The conflicting results may also be ascribed to plant species, environmental and physiological conditions of both stock plants and cuttings after being taken. According to Rasmussen & Andersen (1980), factors such as ABA concentration, rooting period length, and stock plant growth conditions all influenced the response of *Pisum sativus* cuttings to ABA.

It seems that ABA may stimulate rooting in varying ways. First, exogenously applied ABA has generally been found to partially overcome gibberellic acid (GA_3) induced inhibition of rooting (Hartung *et al.*, 1980). This is consistent with the opposing nature of ABA and GA_3 in other developmental phenomena such as bud and seed dormancy. Second, ABA might reduce apical shoot growth in cuttings with leaves, leading to improve rooting by reducing competition for assimilates (Rasmussen & Andersen, 1980). For example, Hartung *et al.* (1980) found that there was an accumulation of assimilates, particularly sucrose, in the base of ABA treated *Phaseolus vulgaris* cuttings and the accumulation of assimilates was associated with increased rooting. Recently, Tari & Nagy (1996) suggested that ABA might stimulate rooting through its effect on ethylene release. In their experiment, ABA treatment restored the rooting of primary leaf cuttings of *Phaseolus vulgaris* treated by paclobutrazol, a triazol growth retardant and an inhibitor of gibberellin biosynthesis.

At the same time, it enhanced ethylene production and the concentration of the precursor 1-aminocyclopropane-1-carboxylic (ACC). This effect of ABA could be abolished by $CoCl_2$, an inhibitor of ACC oxidase.

Cytokinins Application of cytokinins to cuttings generally inhibit adventitious root formation (Jarvis, 1986; van Staden & Harty, 1988). Such inhibitory effects have been reported in the rooting trials of *Acer rubrum* and *Eucalyptus camaldulensis* (Bachelard & Stowe, 1963), *Phaseolus aurens* (Chandra *et al.*, 1973), *Phaseolus vulgaris* (Humhpries, 1960), *Pinus radiata* (Smith & Thorpe, 1975b), *Pisum sativum* (Eriksen, 1974; Bollmark & Eliasson, 1986) and *Zebrina pendula* (Delegher-Langohr, 1974). However, it appears that some cytokinins may be necessary for rooting, as application of small amounts of cytokinins is necessary for root formation in tissue culture. Therefore, it seems that a low level of cytokinin or favourable auxin/cytokinin ratio is good for rooting. This is supported by the study of Eriksen (1974) in *Pisum sativum*. Root formation was inhibited by application of high concentration of BA to the cuttings which were both decapitated and disbudded, whereas root formation was enhanced by similar application of BA to the cuttings which were only decapitated. This may be due to the supply of auxin by shoot buds to the rooting region and their removal could lead to an unfavourable auxin/cytokinin ratio (Jarvis, 1986).

Gibberelins Like cytokinins, exogenously applied gibberelins (GA_3) generally inhibit adventitious root formation (Batten & Goodwin, 1978; Jarvis, 1986; Hansen, 1988). Usually, the inhibition increases with increasing concentrations higher than 10^{-6} M. However, root formation may be stimulated by exogenous application of gibberelins in some plant species under certain environmental conditions. In a review on influence of gibberelins on adventitious root formation, Hansen (1988) gave a list of plant species showing the effects of exogenously application of gibberelins on root formation. Among the 29 plant species listed, the inhibition of root formation by gibberellin was observed in 19 species; a stimulatory effect was observed in 4 species; and in the other 6 species both stimulation and inhibition were observed.

Pacloburazol This is an experimental anti-gibberellin plant growth regulator which exhibits strong growth-control properties in a range of species (Curry &

Williams, 1983; DeJong & Doyle, 1984; Upadhyaya *et al.*, 1985). Interestingly, several studies indicated that this chemical could increase the number of roots formed on cuttings of *Phaseolus vulgaris* and *Plectranthus australis* (Davis *et al.*, 1985) and *Vigna radiata* (Porlingis & Koukourikou-Petridou, 1996; Pan & Tian, 1999). Porlingis & Koukourikou-Petridou (1996) considered that paclobutrazol promoted rooting by interfering with gibberellin biosynthesis but, in addition, it could influence another process or processes. Pan & Tian (1999) suggested that the cause of the synergistic effect of auxin plus paclobutrazol treatment might be due to increased endogenous auxin level, because capillary electrophoresis analysis showed that the endogenous auxin levels in the treatments with auxin plus paclobutrazol were higher than those of auxin alone.

1.3.2 Other chemicals applied to enhance adventitious rooting

Currently, it is generally agreed that auxin plays a central role in the process of root initiation and/or development. However, some other compounds for example some phenolics and polyamines, may also have some promoting effects on rooting. There is substantial evidence that applied phenolics may affect rooting of cuttings (Haissig, 1986; Jarvis, 1986). Phloroglucinol reportedly stimulated rooting and shoot development in tissue cultures of tree species including apple (James & Thurbon, 1981a 1981b; Zimmerman, 1984), pear (Wang, 1991), plum (Jones & Hopgood, 1979; Baleriola-Lucas & Mullins, 1984), *Cinchona ledgeriana* (Hunter, 1979) and cocoa (Passey & Jones, 1983), although the work with apple has shown that this compound may be ineffective (Zimmerman & Broome, 1981). Some other phenolics such as *p*-coumaric, caffeic and chlorogenic acids could enhance rooting when applied alone and increased the effect of exogenous auxin application in cuttings of mung bean (Fernqvist, 1966). That phenolics enhanced the rooting response following auxin application was also observed by other researchers (Basu *et al.*, 1969; Hess, 1969). Therefore, it has been suggested that phenolics act as a cofactor or synergist of auxin during the rooting process.

Studies on the relationship between exogenous application of polyamines and adventitious rooting have also been reported. During the 80's, the effect of the exogenous application of polyamines on adventitious rooting had almost exclusively

been studied in *Vigna radiata* (mung bean) hypocotyl cuttings. Exogenous application of polyamines could stimulate (Jarvis *et al.*, 1983b; Shyr & Kao, 1985) or inhibit adventitious rooting (Friedman *et al.*, 1982). However, since the early 90's, reports on this subject in other species, particularly in woody species, have appeared. In *Prunus avium*, not only decreased rooting, but also at a concentration of 500 μM, spermidine strongly inhibited rooting (Biondi *et al.*, 1990). Putrescine was also observed to decrease rooting in walnut (Rugini *et al.*, 1993). On the contrary, in *Olea europaea* (olive) both in cuttings (Rugini *et al.*, 1990 1991; Rugini *et al.*, 1993) and *in vitro* explants (Rugini & Fedeli, 1990; Rugini *et al.*, 1991), putrescine, in combination with auxin, promoted early root formation and increased rooting percentage. But, in *Castanea sativa* (chestnut), *Prunus dulcis* (almond), *Simmondsia chinensis* (jojoba), and *Prunus armeniaca* (apricot), putrescine did not affect adventitious rooting. Although the findings on effect of exogenous application of polyamines are controversial, some interesting information has emerged regarding polyamines and adventitious root formation (Sankhla & Upadhyaya, 1988). For example, treatment with auxin increased polyamine contents (Altman & Bachrach, 1981; Jarvis *et al.*, 1983b; Geneve & Kester, 1991; Rugini *et al.*, 1991); exogenous application of polyamines had a stimulatory effect on adventitious rooting only in the presence of boron (Jarvis *et al.*, 1983b).

1.3.3 Stock plant conditions

1.3.3.1 Maturation

Ever since the first report on inverse relationship between ontogenetic age and adventitious rooting ability by Gardner (1929), it has frequently been observed that rooting ability of cuttings from many woody plants, especially tree species, decreases with increasing age of seedling-derived mother plants (Lovell & White, 1986). During the process of maturation, the upper and peripheral parts of a plant are the first to exhibit reduced rooting ability. For example, the cotyledonary node has a very high rooting ability but by the 15^{th} node adventitious rooting ability is almost lost in *Eucalyptus* (Paton *et al.*, 1970).

The reasons for loss of adventitious rooting potential with maturation are very poorly understood although many studies have been conducted to explain the loss of adventitious rooting ability with maturation, including anatomical characteristics, endogenous levels of plant growth substances, rooting cofactor and promoters, and rooting inhibitors. There has been little or no convincing evidence that extractable or translocatable factors are physiologically important in explaining loss of adventitious rooting ability with maturation. However, indirect evidence from grafting and co-culture experiments suggest that non-mobile factors such as anatomical characteristics may be important in explaining loss of adventitious rooting ability in some species. (Hachett, 1988).

1.3.3.2 Stock plant environment

The physiological conditions of the stock plant is the result of the interaction between genotype (species and cultivars) and environmental factors (light, water, temperature, O_2, CO_2, and nutrition) (Moe & Andersen, 1988). It is evident that the stock plant environment exerts a strong influence on adventitious rooting in stem cuttings (Hansen, 1975), on adventitious root and bud formation in cuttings with leaves (Heide, 1964 1965), and on regeneration in flower peduncle segments (Appelgren, 1976).

The changes of rooting ability with seasonal variation have frequently been observed for many years (Hartmann & Kester, 1983; Andersen, 1986; Moe & Andersen, 1988). For instance, Palanisamy & Kumar (1997) investigated the influence of seasonal changes on adventitious rooting ability of shoot cuttings in *Pongamia pinnata*. They found that there were two peaks of rooting ability in a year, i.e. March and September and the former was more pronounced than the latter. It has also been noted that the adventitious rooting ability of herbaceous cuttings fluctuates during the seasons even if they are taken from stock plants grown in greenhouses (Moe & Andersen, 1988). Moe & Andersen (1988) concluded that this phenomenon seems to be related to seasonal changes in temperature, irradiance level, and/or interaction between irradiance and photoperiod, suggesting that the physiological status of the stock plant at the time of taking the cuttings is of utmost importance for the rooting process.

The relationship between irradiance levels that the stock plants were exposed to and subsequent adventitious rooting is controversial (Andersen, 1986; Moe & Andersen, 1988). Increase of irradiance level may promote rooting, inhibit or delay rooting, or have no effect on rooting (Moe & Andersen, 1988). Number of roots per cutting increased with the increasing irradiance level from 0 to 100 W/m^2 in *Chrysanthemum morifolium*, while it dropped markedly with the increasing irradiance level from 0 to 70 W/m^2 in *Pisum sativum*. In contrast, adventitious rooting of cuttings was not influenced by the change of irradiance level in apple (Christensen *et al.*, 1980) and *Dahlia* pps. (Biran & Halevy, 1973 1973b). It is evident that stock plants and cuttings require a certain minimal level of light to root well, but this optimal irradiance level may vary from species to species and sometimes from cultivar to cultivar.

Reports on the relationship between adventitious rooting and other stock plant environmental factors such as temperature, water stress, CO_2 and mineral nutrition are rather sparse. Some hints of these subjects were given by Moe & Andersen (1988). In general, cuttings from the stock plant grown at a low temperature (3-4°C) formed more adventitious roots than those did from stock plant grown at higher temperatures (Kaminek, 1962; Appelgren & Heide, 1972). Water-stressing stock plants may stimulate or inhibit subsequent adventitious rooting, whereas waterlogged plants produce cuttings with better rooting ability in some species. The controversy is based on the problems of the interaction between water stress and stock plant's exposure to irradiance and of the abscisic acid and ethylene status of the cuttings caused by stock plant water stress (Moe & Andersen, 1988). CO_2 enrichment of stock plant generally has a positive after-effect on adventitious rooting and lateral branching of the rooted cuttings. This may be because CO_2 enrichment can decrease light compensation point and inhibit photorespiration, resulting in higher carbohydrate level and dry weight. Generally, adventitious rooting is negatively correlated with the content of nitrogen in cutting.

1.3.3.3 Stock plant treatments to obtain cuttings with high rooting ability

Rejuvenation of mature plant tissues by some methods tends to increase adventitious rooting ability. Severe pruning and hedging have been used successfully to obtain

shoots with increased adventitious rooting ability in apple (Hatcher, 1959), *Pinus radiata* (Libby *et al.*, 1972), and *Eucalyptus* pps. (Martin & Quillet, 1974). Grafting mature scions on juvenile rootstocks have been demonstrated that juvenile characteristics and increased adventitious rooting ability were induced in some species (Martin & Quillet, 1974; Franclet, 1979). New adventitious shoots induced on matured stock plants also displayed some rejuvenated characteristics and increased adventitious rooting ability in apple (Stoutemyer, 1937), *Populus tremula*, *P. tremuloides*, and *Ulmus* pps. (Heybroek & Visser, 1976). During *in vitro* propagation, some adult plant tissues could rejuvenate and increase rooting ability. In this case the rooting ability is increased with increasing numbers of subcultures (Shen & Mullins, 1983; Mullins, 1985).

Adventitious rooting ability can also be increased by manipulations of stock plant, including etiolation which is defined as the total exclusion of light, or forcing new shoot growth under heavy shade, and banding which is a pretreatment that excludes light from the portion of the stem that is to become the cutting base (Maynard & Bassuk, 1988). Etiolation and banding increase the portion of stem tissues occupied by undifferentiated parenchyma (Herman & Hess, 1963; Schmidt, 1982), which is thought to be an intermediate in the initiation of adventitious roots (Hartmann & Kester, 1983) and could be associated with the increase in the herbaceous character for improved rooting (Frolich, 1961; Christensen *et al.*, 1980). Physiologically, etiolation may influence auxin metabolism, factors affecting the IAA-O activity, and in the activity of rooting cofactor substances (see review by Maynard & Bassuk, 1988 and references therein).

1.3.4 Controlling the environmental conditions to improve adventitious rooting

Without proper environmental control, it is very difficult to root many types of cuttings (Loach, 1988a). Environmental factors, such as light, CO_2, O_2, pH, water, temperature, and nutrients all can influence adventitious root formation (Andersen, 1986; Loach, 1988a). However, it seems that proper temperature and avoiding water loss of cuttings, particularly cuttings with leaves, by keeping constant humidity are of particular importance to rooting these cuttings.

Several methods can be employed to maintain a proper humidity in rooting ambience. Enclosures are used primarily to maintain vapour pressure of water in the air at near saturation by preventing the escape of water vapour. The major advantage of enclosures are simplicity and low cost, but heat is readily trapped because of the closed system (Loach, 1988a). At present, the most commonly used material is polyethylene film, which has low permeability to water vapour (Dubois, 1978), and is inexpensive. Polyethylene film covered enclosures are widely used both outdoors and in the greenhouse.

Mist propagation originated in the late 1930s and early 1940s, and remains the most widely used propagation system for cuttings with leaves. In this system, vapour pressure in leaf and leaf-to-air vapour pressure gradient remain low and therefore water loss is restricted. This helps to maintain the cuttings in a turgid condition. Advantages of this system is that the cuttings are immediately accessible and the propagator can easily view their conditions and attend to any obvious problem (Loach, 1988a).

Fog systems aim to maximize vapour pressure of water in the air, i.e. raise the ambient humidity by using fog generators producing very fine droplets of water (<20 μm) (Loach, 1988a). The advantages in this system are the fine water droplets remain airborne for a relatively long period; the surface/volume ratio of water is high as the water is finely divided; and leaching of nutrients and over-wetting are avoided as the water passes into the air as vapour rather than falling on to the surfaces of leaves and rooting medium.

In addition, rooting media and sanitation in the rooting ambience also affect adventitious rooting of cuttings.

1.4 Root induction by *Agrobacterium rhizogenes*

1.4.1 Agrobacterium rhizogenes *and its hosts*

Agrobacterium rhizogenes, a soil inhabiting bacterium, is a natural plant pathogen responsible for adventitious 'hairy root' formation at the site of infection (Strobel &

Nachmias, 1988; Nilsson, 1995). The phenomenon of 'hairy root' was first described by Riker (1930). *A. rhizogenes* and the 'hairy root' disease had not been given much attention until 1970s. This was probably due to no dramatic crop loss and pathological symptoms in plants infected by this bacterium and the time was not ripe for molecular biologists to study this phenomenon (Strobel & Nachmias, 1988).

In the early 1970s, several reports suggesting *A. rhizogenes* producing 'super rooting' hormones in liquid media were published (Strobel & Nachmias, 1988). However, other studies indicated that the intact bacterium was required to induce rooting (Moore *et al.*, 1979; Strobel & Machmias, 1985).

Ackermann (1977) successfully regenerated tobacco shoots from 'hairy roots' induced by *A. rhizogenes*. These shoots grew to be fertile plants, but displayed a high aberrant phenotype in that they were dwarfs with extremely short internodes and a large number of wrinkled leaves. This phenotype proved to be heritable and occurred in all the following generations. This led Ackermann to suggest that the generated plants had been genetically transformed with genes from *A. rhizogenes*. Later, it was indicated that the hairy roots contain DNA from *A. rhizogenes* (Chilton *et al.*, 1982; Spanò & Costantino, 1982; White *et al.*, 1982; Willmitzer *et al.*, 1982).

Before the late 1980s, it had been considered that only dicotyledonous plants could be infected by *A. rhizogenes* but not mono- or polycotyledonous plants (De Cleene & De Ley, 1981; Strobel & Nachmias, 1988). However, cross-inoculation tests have shown that the possible host-range is much wider. Since the early 1990s, some reports on root induction in monocotyledonous plants (Porter, 1991) and polycotyledonous plants (McAfee *et al.*, 1993; Tzfira *et al.*, 1996b) by *A. rhizogenes* have appeared. For example, Tzfira *et al.* (1996b) used *A. rhizogenes* stain LBA9402 to transform *Pinus halepensis* embryos, seedlings and shoots, all of which expressed GUS activity. Polymerase chain reaction (PCR) and Southern blot analyses confirmed the *uidA*-transgenic nature of the root and callus, as well as the presence of *rolB* and *rolC* genes in roots from infected seedlings.

Although intact *A. rhizogenes* (living cells) is required to induce 'hairy roots' (Moore *et al.*, 1979; Strobel & Nachmias, 1985) as stated above, occasionally, T-DNA

of the bacterium could not be detected in the roots produced in response to bacterial infection (McAfee *et al.*, 1993). This suggests that no T-DNA transfer had occurred or that it was present at a level lower than that detectable by the Southern blot procedure (McAfee *et al.*, 1993). After opine and molecular analysis for TR- and TL-DNA, Bassil *et al.* (1991) were unable to determine if the roots induced on hazelnut cuttings contained transformed tissue. Simpson *et al.* (1986) pointed out that in some species many roots produced by *A. rhizogenes* were not transformed. These findings suggest that the bacterium could induce roots by modifying the root environment, such as secretions of hormones, and organic acids, for example, malic, lactic, fumaric, citric acids, which favour rooting (McAfee *et al.*, 1993).

1.4.2 The Ri plasmid and rol*-genes*

That the discovery of the pathogenic strains of *Agrobacterium* contained giant plasmids (Zaenen *et al.*, 1974), which were essential for tumour induction, allowed very rapid advances in our understanding of the molecular mechanisms underlying the *Agrobacterium* infection process. (Nilsson, 1995). The large (*ca.* 200kb) plasmid, called root-inducing (Ri) in *A. rhizogenes* contain most of the functions needed for DNA transfer. Particularly, two regions are important: the T-DNA, which represents the DNA that is copied and actually transferred to the plant cells; and the virulence (*vir*) region, which encodes most of the trans-acting factors that are needed for this transfer to occur.

In the T-DNA region, there are four genes defined as *rolA-D* which are involved in root formation and morphology (White *et al.*, 1985). The individual *rol* genes were isolated and transferred into plant cells. The gene *rolA* (300 bp) encodes a very basic 100-amino acid protein with molecular weight of 11 kDa (Levesque *et al.*, 1988). One of the most typical characters of transformed tobacco plants with *A. rhizogenes* is severely wrinkled leaves, stunted with extremely shortened internodes and had small rounded leaves. This is ascribed to the expression of *rolA* (Schmülling *et al.*, 1988; Sinkar *et al.*, 1988). Transgenic tobacco protoplasts expressing the *rolA* gene were 30 to 1000 times more sensitive to auxin than wild-type (Maurel *et al.*, 1991).

The gene *rolB* (777 bp), encoding a 259-amino acid protein with a molecular weight of 30 kDa (Levesque *et al.*, 1988), seems to have a central role since it is the only *rol*-gene on its own that is able to induce hairy roots in tobacco (Spena *et al.*, 1987) and carrot (Capone *et al.*, 1989). Transgenic tobacco protoplasts expressing the *rolB* gene from its endogenous or 35S promoter are 3,000 to 100,000 times more sensitive to auxin than wild-type (Maurel *et al.*, 1991, 1994), suggesting that *rolB* is responsible for an auxin like activity (van der Salm *et al.*, 1997).

The gene *rolC* (540 bp), encoding a 180-amino acid protein with a molecular weight of 20 kDa, is the least effective of the *rol*-genes in inducing increases in auxin sensitivity (Maurel *et al.*, 1991). In contrast, the expression of *rolC* gene seems to increase cytokinin activity (Schmülling *et al.*, 1988 1993), suggesting that *rolC* has a cytokinin like action such as diminishing apical dominance (Fladung, 1990; van der Salm *et al.*, 1997).

The expression of *rolD* gene produced an increased amount of callus as well as attenuated root growth (White *et al.*, 1985). However, information on *rolD* gene is very limited.

1.4.3 *Application of* Agrobacterium rhizogenes

1.4.3.1 Genetic engineering vector

Like *A. tumefaciens*, *A. rhizogenes* can be used as genetic engineering vector to transform some plant species. For some difficult-to-root species, *A. rhizogenes* itself carries useful genes, i.e. *rol* genes, *iaaM* and *iaaH* genes (i.e. the two genes encoding enzymes for auxin biosynthesis). Rooting ability of the plant regenerated from the transformed roots and their following daughter generations may be increased. For example, van der Salm *et al.* (1997) used *A. rhizogenes* for the production of genetically modified rose rootstocks with an improved root system. On the other hand, genes encoding 'desirable' characteristics could be inserted into the T-DNA of Ri plasmid in *A. rhizogenes* which are subsequently expressed in regenerated plants from the transformed roots.

1.4.3.2 Promoting adventitious rooting of cuttings

Several approaches have been adopted to promote adventitious root formation in difficult-to-root species. *A. rhizogenes* has been increasingly used to enhance ornamental vegetative propagation (Hatta *et al.*, 1996). *A. rhizogenes* were used to induce roots in a difficult-to-root subclone of apple (Patena *et al.*, 1988) and for propagation of hazelnut cuttings (Bassil *et al.*, 1991). An improvement in root number, quality and/or rooting percentage was observed on cuttings inoculated with *A. rhizogenes* in almond tree (Strobel & Nachmias, 1985), olive tree (Strobel *et al.*, 1988), jujube tree (*Ziziphus jujuba* Mill.) (Hatta *et al.*, 1996), *Pinus banksiana* and *Larix laricina* (McAfee *et al.*, 1993). In this case, however, the promotion of rooting was not always due to transferring T-DNA of *A. rhizogenes* to the plant genome as described above, but the bacterium seems to have improved the rooting environment.

1.5 Future approaches in adventitious root formation

Although plant propagators have been successful in manipulating rooting of cuttings of many plant species, there are still many species, particularly in their mature stage of development, which cannot be successfully propagated from cuttings on a commercial scale (Hachett & Murray, 1994). And the past physiological and biochemical studies on adventitious roots have largely been aimed at gaining insights regarding the influences of plant growth regulators at the metabolic levels. Therefore, the fundamental biology of this important developmental phenomenon, adventitious root formation, is still obscure (Haissig, 1988; Haissig *et al.*, 1992).

In order to further improve adventitious rooting, a better understanding of the mechanisms that control the root initiation process is required (Haissig, 1988; Haissig *et al.*, 1992; Hachett & Murray, 1994). Haissig (1988) pointed out that it is the time to redirect the adventitious rooting research, in which the past physiological and biochemical studies should be strategically used to guide future research. To do this, "it seems that we must move closer to the gene level" (Haissig *et al.*, 1992). Molecular genetic studies on adventitious root formation, with high sensitivity, have only been initiated recently and may provide new insights into the control of this developmental phenomenon. To begin the investigation on the molecular control of adventitious root

formation, an understanding of the transcriptional events during the initiation of root primordia is needed (Chen *et al.*, 1996). There are three basic molecular approaches that may be used to study rooting-related genes (Haissig *et al.*, 1992).

Firstly, target genes that are known or at least likely to be related to rooting (e.g. some genes encoding potential biochemical maker of adventitious rooting such as PO and PPO) could be cloned using heterologous (from different species) probes, or these genes could be identified immunologically by using highly purified gene products extracted from the target species. Then the cloned genes could be characterized, and the gene expression and function could be determined by recombinant DNA methods. The relationship, if any, between the selected gene and rooting could be established by empirical recombinant DNA experimentation.

Secondly, unknown rooting-related genes could be isolated by differential screening between plant materials that contrasted phenotypically in rooting response. Such searches for genes and mRNA are becoming possible because of the development of powerful techniques for amplification of nucleic acid molecular species in a population. For example, PCR (polymerase chain reaction) may achieve a more than 10^6-fold amplification of DNA molecules *in vitro* in a few hours (Ehrlich, 1989; Wang *et al.*, 1989). The resolution of amplification-based analytical methods for RNA is great enough so that developmentally specific RNAs can be detected from one or few cells (Brenner *et al.*, 1989; Wang *et al.*, 1989) by subtractive hybridization technologies (e.g. Travis & Sutcliffe, 1988). With regard to adventitious rooting, this kind of work is beginning and very limited. van der Krieken *et al.* (1991) firstly isolated and identified genes with changes in their expression during the rooting process of tissue cultured apple cuttings. They compared gene expression in rooting and non-rooting tissues by the construction and differential screening of cDNA libraries. Then, Sussex *et al* (1995) used this kind of technique to examine molecular and cellular events associated with the initiation of lateral root formation from differentiated pericycle cells in radish and *Arabidopsis*. They isolated 51 different gene sequences from subtracted cDNA libraries made at the stages of lateral root formation. Chen *et al.* (1996) isolated two genes that were associated with auxin-induced adventitious root formation in mungbean (*Vigna radiata*) using differential

screening of cDNA library. Recently, Butler & Gallagher (1999) reported that an adventitious rooting-related oxygenase gene was isolated and characterized in apple (*Malus domestica*). More interestingly, in loblolly pine (*Pinus taeda*) a gene encoding α-expansin, which was strongly expressed during adventitious root formation, was obtained using differential display RT-PCR.

Thirdly, genotypic variation in rooting response would be related to molecular structure by analysis of restriction fragment length polymorphisms or other molecular features such as transposable elements. Genes related to rooting would be identified based on their close physical linkage to markers, or by molecular 'tagging' with transposable elements or inserted foreign DNA oligomers.

In addition, comparisons between easy- and difficult-to-root cultivars, between mutants incapable of rooting and the wild-types, based on molecular approaches, may provide important information for rooting mechanism. In this area, *Arabidopsis* is a good research material as many mutants exist in this species. Stable high (HA) and low (LA) adventitious rooting lines have been reported (King & Stimart, 1998). Three different categories of mutants for lateral root formation have been described, i.e. the mutants that form too many lateral roots, those initiate lateral roots but fail to mature, and those do not produce lateral roots (Smith & Fedoroff, 1995). The mutants incapable of adventitious root formation may be selected for studying the molecular control of adventitious root formation (De Klerk, 1996). *A. rhizogenes* mediated genetic transformation, the natural genetic engineer of rooting, is another good candidate to study the rooting process.

1.6 Adventitious root formation in *Pinus radiata*

Pinus species are the most widely grown trees in commercial forestry around the world, supplying, with other conifers, the world's main sources of plantation-grown wood. *Pinus radiata* D. Don (syn. *P. insignsis* Douglas), usually called radiata or Monterey pine, is one of the most economically important species grown extensively in several southern-hemisphere countries, such as New Zealand, Australia, Chile, and South Africa (Smith & Thorpe, 1975a; Walter *et al.*, 1994). Vegetative propagation

plays an important part in the genetic improvement of *P. radiata* in New Zealand (Thulin & Faulds, 1968; Reilly & Washer, 1977). Using rooted cuttings of *P. radiata* for direct afforestation has been advocated by Jacobs (1939) and Fielding (1964) for many years as it is very desirable to obtain uniformity in quality both within and between trees by vegetative propagation (Thulin & Faulds, 1968).

Since the 1930s many researchers have conducted experiments on rooting cuttings of *P. radiata*. In New Zealand, many investigations have been continuously carried out largely at Forest Research Institute in Rotorua since the 1950s. These scientists hoped to greatly improve the rooting response of the cuttings of this species. Subsequently, a number of investigations indicated that the cuttings of *P. radiata* from young trees, particularly less than 10 years old, can be rooted relatively easily, but that the rooting ability decreased quickly with the age of stock trees (Jacobs, 1939; Allsop, 1950; Pawsey, 1950; Fielding, 1954 1964; Libby & Conkle, 1966).

In addition to the stock plant age, other factors also affect adventitious root formation of *P. radiata* by cuttings. It was reported that the health of the tree was more important than the age of stock tree, and that the rooting ability depends, to a largely extent, upon the amount of food (carbohydrates, proteins, etc.) reserves present (Libby & Conkle, 1966). This led to the suggestion that in *P. radiata* low rooting ability of the cuttings from old trees is possibly because the food reserves is insufficient to support the metabolic activity during the period of root formation (Cameron, 1968). Thulin & Faulds (1968) also reported that the best cuttings for rooting were those from branches grown in full sunlight, with dark green, and healthy foliage.

Work on root induction in cuttings of *P. radiata* indicated that pre-severance, which may condition the cuttings to uniform physiological status, could increase the rooting ability (Cameron, 1968). Jacobs (1939) found that the cuttings rooted more easily if the shoots, from which the cuttings were made, had been partly broken while still attached to the tree for some time before actual removal. Girdling, as a pre-severance treatment to induce callus, also significantly improved rooting of the cuttings of *P. radiata*. This treatment was considered as a combination of nutritional and hormonal effects, since the removal of a band of phloem tissue blocks the

basipetal movement of photosynthetic products and auxin which accumulate at the base of the cutting (Cameron, 1968).

Seasonal influence on rooting response of *P. radiata* cuttings has also been investigated. Most workers (Jacobs, 1939; Mirov, 1944; Pawsey, 1950) demonstrated that the cuttings collected in autumn and winter rooted better than those did in spring, when the branches grew vigorously. The effect of cold-stored cuttings of *P. radiata* prior to rooting is controversial. Libby & Conkle (1966) reported that cold storage for 20-50 days might actually be beneficial, increasing the speed of rooting and the number of roots per cutting. However, Horgan & Holland (1989) later indicated that the shoots that had been cold-stored prior to rooting did not survive well.

As regards to the effect of auxin, reports by Melchior (1963), Libby & Conkle (1966), Hoffman & Kummerow (1966), Kummerow (1966) indicated that both rooting rate and root number of *P. radiata* the cutting were increased by the treatment with IBA. Surprisingly, other workers (Jacobs, 1939; Allsop, 1950; Audus, 1953) found that auxin did not assist in adventitious rooting or had slight promotive effect in *P. radiata*. Cameron (1968) considered that this was due to the natural variation in hormones and growth inhibitor in the cuttings before removal.

Smith & Thorpe (1975a, 1975b, 1977) investigated the anatomical process and the effects of applied chemicals, including phytohormones and phenolics, on adventitious root initiation in *P. radiata* by using de-rooted seedling as a model system. The anatomical study showed that adventitious root initiation in hypocotyls of *P. radiata* consisted of three distinct phases, i.e. pre-initiative phase, a histologically silent stage - no histological changes after the cuttings being made; initiative phase, beginning with swelling of a single parenchymatous cell followed by cytoplasmic migration and asymmetric division; post-initiative phase, continuing division of derivatives of the peripheral cells to form meristemoids which subsequently develop into root primordia. It seemed that IBA was required during the entire rooting process. They concluded that "such a bimodal effect of an exogenous auxin in adventitious root initiation does not appear to have been previously reported". Kinetin markedly inhibited the root initiation when it was applied during the pre-initiative phase, but the inhibitory effect escaped after the formation of meristemoids. GA_3 may stimulate or inhibit rooting depending on the

time of application. The effects of phenolics on rooting largely depend on the response of the cuttings to IBA alone and the initiative stage of root primordia. When rooting by IBA alone was low, phenylalanine, tryosine, chlorogenic acid and ferulic acid, in the presence of IBA, stimulated rooting, particularly applied during the root initiative stage. However, the phenolics had no effect on or even inhibited rooting when rooting by the IBA alone was high.

More recently, tissue culture has been employed for vegetative propagation of *P. radiata* (Reilly & Washer, 1977; Horgan & Aitken, 1981; Aitken-Christie *et al.*, 1985; Horgan & Holland, 1989). Adventitious roots were induced most consistently from the adventitious shoots initiated from embryonic tissue on a modification of GD medium (Gresshoff & Doy, 1972) containing 2.0 mg/l IBA and 0.1 mg/l NAA, which made the shoots greener (Reilly & Washer, 1977). A combination of tissue culture method and traditional vegetative propagation method was also used to produce *P. radiata* plants. Shoots initiated from tissue culture as the cuttings were pre-treated with auxin and then were induced to root on mixed soil medium (Horgan & Aitken, 1981; Aitken-Christie *et al.*, 1985; Horgan & Holland, 1989). A reliable method of rooting shoots from micropropagated *P. radiata* was established (Horgan & Holland, 1989), averaging 78% rooting. This is a combination of removal of callus from the base of the cutting prior to an auxin treatment, pre-rooting treatment of nutrient medium containing 6% sucrose, and inducing roots on a free-draining peat-pumice-perlite medium and under controlled environment conditions.

1.7 The objectives of this study

Rooting is an important phenomenon for horticulture, agriculture and forestry from an economic point of view, while it is a highly interesting developmental pathway from a scientific point of view (De Klerk *et al.*, 1999).

The purpose of this study was primarily investigation of biochemical and molecular events associated with specific stages in the process of adventitious root formation in *Pinus radiata*. The reasons why *P. radiata* was chosen for this study are because this species is economically important and because no work has been carried

out on the biochemical and molecular aspects of adventitious root formation. Varying plant tissues, including hypocotyls (Smith & Thorpe, 1975a 1975b 1977), mature shoots (Thulin & Faulds, 1968), and micropropagated shoots from embryos (Reilly & Washer, 1977; Christensen *et al.*, 1980; Horgan & Aitken, 1981), seedling shoot tips (Horgan & Aitken, 1981) and mature shoots (Horgan & Holland, 1989), were used as plant materials to induce adventitious roots. Furthermore, such a study may be useful for further improving root formation in cuttings of *P. radiata.*

With 3 considerations in mind, a reliable system was required for the investigations of biochemical and molecular aspects during adventitious root formation. Firstly, many past studies were carried out under non-aseptic conditions, under which it was not possible to rule out the unpredictable interference from microbial metabolism. Secondly, to avoid studying root formation in starving or senescent tissue, a basal nutrient medium was considered to be appropriate. Thirdly, a preliminary study showed that the hypocotyls of de-rooted *Pinus radiata* seedlings responded and grew well in IBA-containing nutrient medium.

Rooting seems to be mediated through a chain of biochemical reactions in competent cells (Bhattacharya, 1988). The biochemical reactions are likely to involve in an alteration of gene expression (Dhindsa *et al.*, 1987; Kang, 1995). However, very little information is available about changes at the gene expression level (Macisaac & Sawhney, 1990; Kang, 1995), and none in *Pinus radiata.* Therefore, protein changes, including protein contents and protein patterns of accumulation in plant tissues by both 1D SDS- and 2D-PAGE, were investigated under root-forming and non-root-forming conditions. The aim was to generally understand protein change during rooting process and find rooting specific protein markers in *P. radiata.*

Some enzymes are directly or indirectly associated with adventitious root formation. The activities of PO, PPO, amylase, IAA-O and SDH were determined with tissue extracts and at the histological level. In addition, changes in starch and soluble sugars were determined in different root-forming treatments.

More sensitive molecular techniques were employed in order to further identify the molecular events that are associated with adventitious root formation. The rooting region of IBA-treated hypocotyls (rooting treatment) was chosen as the source of

tissue for cDNA synthesis in an attempt to isolate gene sequences that are expressed during the early stage of root initiation. An enrichment of rooting-specific transcripts was achieved by the subtraction of messages to control (non-rooting) treatment. Polymerase chain reaction (PCR) technology was applied to amplify the subtracted cDNA because of the small quantity of the cDNA remaining after subtraction.

Since the early 1990s, some reports on root induction by *A. rhizogenes* in monocotyledonous plants (Porter, 1991) and polycotyledonous plants (McAfee *et al.*, 1993; Tzfira *et al.*, 1996b) have appeared. However, there is no report on root induction by *A. rhizogenes* in *Pinus radiata.*

In summary, the objectives of this study are:

1) To establish a reliable rooting system for the further biochemical and molecular studies.

2) To identify potential biochemical markers, e.g. proteins, enzymes and carbohydrates for adventitious root formation.

3) To initiate a molecular study in radiata pine towards the isolation of genes specifically expressed during adventitious root formation in the cuttings.

4) To evaluate the effects of *Agrobacterium rhizogenes* on adventitious root formation in *Pinus radiata.*

The overall aim of this study is to increase our understanding of the critical events leading to the formation of adventitious roots in radiata pine cuttings. This is a pre-requisite for further studies on the whole question of why some materials (e.g. due to clonal differences or age) form adventitious roots more easily than others do.

Chapter 2

Materials and Methods

2.1 Reagents

The key chemicals were obtained from six companies: ***BDH*** chemicals Ltd, Pool, England; ***BIO-RAD***, Hercules, CA, USA; ***Roche Molecular Biochemicals (BOEHRINGER MANNHEIM,*** *GmbH,* Germany); ***Invitrogen***® Corporation, Carlsbad, CA, USA; ***LIFE TECHNOLOGIES***™, Gaithersburg, MD, USA; and ***SIGMA***®, St. Louis, MO, USA.

BDH*:* 3,5-dinitrosalicylic acid; acrylamide; chloroform; EDTA-Na; formaldehyde; formamide; glycerol; hydrogen peroxide; isopropyl alcohol (iso-propanol); β-mercaptoethanol; N,N'-methylene bisacrylamide; potassium sodium tartrate (Rochelle salt); Resolyte pH 4-8; SDS; sodium acetate; sodium chloride; sodium succinate; starch; Tween-20.

BIO-RAD*:* agarose; PDA; protein molecular weight standard marker.

Roche Molecular Biochemicals (BOEHRINGER MANNHEIM, *GmbH*): Deoxynuleoside Triphosphate (dNTP) Set; DIG DNA Labelling and Detection Kit; DNA molecular weight marker II; *Eco*RI restriction enzyme; kanamycin; mRNA Isolation Kit; nylon membrane (positively charged); *Taq* DNA polymerase; terminal transferase; Magnetic Particle Separator.

LIFE TECHNOLOGIES™***:*** buffer saturated phenol; *Eco*RI PCR primer (5'-ATG/TGC/TCC/AGG/CCG/CTC/TGG/ACA/AAA/TAT/GAA/TTC/$T_{(24)}$-3'); RIZOL® Reagent.

Invitrogen®: Subtractor® Kit; TA Cloning® Kit.

SIGMA®: 2,3,5-triphenyl-tetrazolium chloride; 2D Pharmalyte pH 3-10; ascorbic acid; benidine hydrochloride; CHAPS; DCP; *o*-dianiside; DOPA; DTT; EGTA; glycine; guaiacol; IAA; IBA; kinetin; leupeptin; α_2-macroglobulin; maleic acid (sodium salt); neotetrazolium chloride; N-lauroylsarcosine; Nonidet P-40; protamine sulfate; pyrogallol; sodium citrate; Tris (TRIZMA®); urea; X-gal.

2.2 Rooting system

2.2.1 Plant material

Seed used in all experiments was collected in 1995 from the same population of open-pollinated *Pinus radiata* D. Don trees grown in Canterbury, New Zealand. Seeds were surface-sterilized in 70% (v/v) ethanol for 30 seconds, rinsed briefly in sterile water and then soaked in 30% (v/v) of a commercial bleach (31.5 g/l active sodium hypochlorite) for 30 minutes. After being rinsed in running water overnight, the seeds were surface-sterilized again using 5% (v/v) hydrogen peroxide for 10 minutes. Fifteen of the sterilized seeds were sown in a tissue culture jars (250 ml, clear polycarbonate plastic container from Labserv, Biolab, New Zealand), which was filled with 2/3 capacity of moist, autoclaved vermiculite, and stratified in a cold room (4°C) for one week. The jars were then maintained in a warm dark room (26°C) till seedling emergence before transfer to a plant growth room at 22°C with continuous lighting at 80 $\mu E \cdot m^{-2} \cdot s^{-1}$.

2.2.2 De-rooted seedling cuttings

Uniform seedlings of the same age (1 day after the cotyledons expanded), hypocotyl diameter (about 1 mm) and hypocotyl height (2-2.5 cm) were selected to prepare cuttings as follows. Cuttings were aseptically made at 2.5 cm below the cotyledonary nodes and always within hypocotyl tissues and above the parts of original roots. The original roots of the seedlings were discarded.

Seven de-rooted seedlings were placed in a 250 ml tissue culture jar and were cultured upright with cut-ends of the hypocotyls inserted into various media according to the research objectives in the same plant growth room as described in Section 2.2.1.

The establishment of a reliable rooting system for this research involved testing different basal plant tissue culture media, IBA concentrations, IBA treatment times and sucrose concentrations.

2.2.2.1 Media

GD (Gresshoff & Doy, 1972) modified by Reilly & Washer (1977), RIM (Rancillac *et al.*, 1982), MS (Murashige & Skoog, 1962), ½ MS and 1/5 MS (including mineral salts and organics), ½ MS+ (half strength of major mineral salts and full strength of the other ingredients). When media were examined, IBA and sucrose concentrations were 9 mg/l and 20 g/l, respectively. All media were set at pH 5.8, solidified with 8 g/l agar and then autoclaved for 14 minutes at 137 kPa.

2.2.2.2 IBA treatments

The base of de-rooted *Pinus radiata* hypocotyl cuttings was exposed to media for different times (2, 4, 6, 8 and 10 days) with different IBA concentrations (0, 3, 6, 9, 12 and 15 mg/l). The basal medium was ½ MS supplemented with 20 g/l of sucrose.

2.2.2.3 Sucrose concentrations

The base of de-rooted *Pinus radiata* hypocotyl cuttings was exposed to ½ MS medium supplemented with 9 mg/l of IBA and different sucrose concentrations (0, 10, 20 and 30 g/l) for 10 days.

2.2.3 The effect of kinetin

The effect on root formation and some biochemical changes of kinetin at 10 mg/l, added either alone or in combination with 9 mg/l IBA to the basal medium (1/2 MS medium plus 20 g/l sucrose), was studied. The basal medium without any phytohormone was included as control.

2.2.4 Evaluation of root formation

Rooting efficiency (percentage of rooted cuttings) and number of roots formed per cutting were recorded at day 30 after the start of the root formation experiment. Time of first root appearing was also noted.

2.3 Developmental sequence of root formation: A light microscopy study

2.3.1 Fixation

The lowermost 0.5 cm part of the cuttings (referred to as 'samples' here) were taken daily from day 0 to day 13 after culturing the de-rooted seedling cuttings. The samples were fixed in a 2.5% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.0), and the vials containing the samples were placed in a dessicator under vacuum overnight.

2.3.2 Dehydration

After several washes in the same buffer solution, the samples were dehydrated in a series of graded ethanol: 50, 70, 80, 95, 100 and 100% (v/v).

2.3.3 Infiltration

The samples were infiltrated in a refrigerator for two weeks with liquid resin containing hydroxyethyl-methacrylate resin (Kulzer and Co., GmbH, Wehrheim, Germany), a preparation solution of 100 ml Technovit 7100 and 1 g 'hardener I' (Kulzer and Co., GmbH, Wehrheim, Germany). During this period of time, the samples and resin were gently stirred every two days.

2.3.4 Embedding

The samples were embedded in white resin, a mixture of the preparation solution (same as Section 2.3.3) and 'hardener II' (15/1, v/v, a little amount was mixed up each time because the usable life of this mixture is only 4 minutes, Kulzer and Co., GmbH, Wehrheim, Germany). About 0.3 ml of the white resin was poured in every mould

and the sample was properly positioned. After polymerisation (*ca.* 20 minutes), yellow resin, Technovit 3040 (Kulzer and Co., GmbH, Wehrheim, Germany), was poured into the moulds up to a level of about 2 mm above the base of the moulds. After a period of 5-10 minutes, the samples were removed from the moulds and ready for sectioning.

2.3.5 Sectioning

Transverse sections of 8 μm were cut on a microtome (Reichert Rotocut 2000EX) equipped with glass knives. Sections were mounted in distilled water on slides and allowed to dry overnight, stained with 1% (w/v) Azure II and 1% (w/v) methylene blue and allowed to dry overnight again, then covered with coverslips after Depex mounting medium was applied on the slides.

2.3.6 Light microscopy

Observation of the prepared slides and photographs were taken with an Olympus microscope (model BH2-UCD, Japan) equipped with an Olympus C-35AD-4 camera and an Exposure Control Unit. Kodak reversal film was used.

2.4 Fresh weight changes

The samples (same as Section 2.3.1) were harvested for analysis of fresh weight changes, protein changes, enzyme activity changes and carbohydrate changes because adventitious roots were formed in this part. At least 100 segments from each treatment were harvested for fresh weight determinations.

2.5 Protein changes

2.5.1 Extraction of total proteins

Appropriate amount of samples (same as Section 2.3.1) was placed in a mortar, frozen in liquid nitrogen and ground to a fine powder with a pestle. Or, if applicable, samples were collected in Eppendorf tubes, immediately frozen in liquid nitrogen and stored at -80°C. Then the samples were ground to a fine powder as above when

required. The powder was transferred to pre-chilled Eppendorf tubes and different extraction buffers were added in different experiments.

To run 1D SDS-PAGE, proteins were extracted with a buffer [0.5 M Tris-HCl (pH 8.6), 50 mM EDTA-Na, 0.1 M KCl and 2% (v/v) β-mercaptoethanol (Oliver *et al.*, 1994)] at a ratio of 1:1 (fresh weight of plant tissue : volume of the buffer) in Eppendorf tubes. The homogenates were intermittently vortex mixed for 5 minutes and then centrifuged at 13,000 rpm (4°C) for 15 minutes. Aliquots of the supernatants were analysed on polyacrylamide gels.

The procedure of protein extraction for 2D PAGE was similar to Burritt (1992) with a little modification. 2D-MH extraction buffer (Mayer *et al.*, 1987) modified by Burritt (1992) (Appendix A-1) was added in Eppendorf tubes at the same ratio as described above. The homogenates were intermittently vortexed for 15 minutes and then centrifuged at 13,000 rpm (1°C) for 15 minute. The supernatants were transferred to new Eppendorf tubes and incubated on ice with protamine sulfate (1-2 mg/ml) for 15 minutes with intermittently vortex mixing. After centrifugation at 15,000 rpm (1°C) for 15 minutes, the supernatants were transferred to new Eppendorf tubes again, and urea was added to saturation. The samples were divided into aliquots containing 20 μg protein and stored at -80°C until required or loaded on IEF gels immediately if applicable.

2.5.2 Determination of protein content

The protein contents of the extracts for 1D SDS-PAGE were determined using the original Bradford method (Bradford, 1976). The protein concentrations of extracts for 2D PAGE were assayed by using the modified Bradford method (Ramagli & Rodriguez, 1985). This method reportedly enables an accurate estimation of the protein concentrations of the extracts without interference by ampholyte and without precipitation of the proteins (Ramagli & Rodriguez, 1985). It was found necessary, however, to assay the protein contents prior to the addition of protamine sulphate and urea to the extracts, as both interfered even with the modified method. For this reason, aliquots of each extraction were maintained without adding these chemicals for the protein content to be assayed (Burritt, 1992).

2.5.3 One-dimensional SDS-PAGE

2.5.3.1 Apparatus

PROTEAN™ II chamber (*BIO-RAD*) was employed for SDS-PAGE separation. Power was supplied by a Model 3000xi Computer Controlled Power Supply (*BIO-RAD*). Gels (160 × 200 × 1.5 mm) were cast on the casting stand device (*BIO-RAD*).

2.5.3.2 Gel preparation

Uniform 12% T (w/v) gels were prepared according to Smith (1984). Separating gels were prepared by adding 10% (w/v) ammonium persulfate and TEMED to degassed mixture of stock acrylamide solution and separating gel buffer, and gently swirling to mix thoroughly. The gel solution (Appendix A-2) was poured into the glass plate sandwiches and overlaid gently with distilled water.

After polymerization of the gels for 1 hour, the tops of the gels were extensively washed with distilled water and then dried carefully by using filter paper. Similarly, stacking gels were prepared by adding 10% (w/v) ammonium persulfate and TEMED to degassed mixture of stock acrylamide solution and stacking gel buffer, which were gently swirled to mix thoroughly. Then the stacking gel solution (Appendix A-3) was poured on the top of the separating gels and combs were inserted. The stacking gels were allowed to polymerize for 30 minutes. Two gels were prepared and run together using the *PROTEAN™ II* chamber (*BIO-RAD*).

2.5.3.3 Loading samples and running conditions

A 2× SDS sample buffer (Ausubel *et al.*, 1989) (Appendix A-4) was added at a ratio (v/v) of 1 part buffer to 4 parts protein extract (Section 2.5.1) and the mixture was boiled for 5 minutes. After pouring electrode buffer (Hochstrasser *et al.*, 1988a) (Appendix A-5) into buffer chambers, the prepared samples were loaded into the wells (5 μg protein/well) containing the electrode buffer with micropipette tips.

Gels were run at 50 mA/gel constant current during the stacking phase, after which the current was reduced to 30 mA/gel constant current and run until the tracking dye just reached the bottom of the gels. No cooling was employed.

2.5.4 Two-dimensional PAGE

2.5.4.1 Apparatus

The isoelectric focusing (IEF) separation was performed in the *PROTEAN™ II* chamber (*BIO-RAD*) using the *PROTEAN™ II* tube gel adaptor; the *PROTEAN™ II* chamber was also employed for the second SDS-PAGE separation. Power was supplied by a Model 3000xi Computer Controlled Power Supply (*BIO-RAD*). The first dimensional capillary tubes (1.5 mm I.D. × 180 mm length) were set in Tube Gel Casting Stand (*BIO-RAD*); the second dimensional SDS gels (160 × 200 × 1.5 mm) were cast on the casting stand device (*BIO-RAD*).

2.5.4.2 Preparation of IEF gels

IEF gel solution was prepared as per Burritt (1992) based on Hochstrasser *et al.* (1988a) (Appendix A-6). PDA was utilized as a cross-linking agent instead of N,N'-methylene bisacrylamide (Hochstrasser *et al.*, 1988b) and 2D Pharmalyte pH 3-10 and Resolyte pH 4-8 were used in place of ampholytes pH 3.5-10 and 5-7 used in the procedure of Hochstrasser *et al.* (1988b). The gel solution was filtered through a 0.22 μm nitrocellulose membrane (Millipore Corporation, Bedford, MA 01730, USA), degassed and kept as 1 ml aliquots at -80°C; no degassing prior to use was necessary after this treatment (Mayer *et al.*, 1987). Four μl of 10% ammonium persulfate per millilitre was added to start polymerization. No TEMED was added.

The gels were polymerized in glass capillary tubes (1.5 mm I.D. × 180 mm length, *BIO-RAD*) for at least 3 hours. Tubes were filled to 145 mm with a *BIO-RAD* tube gel loading needle and overlaid with distilled water during polymerization. This was to ensure uniform gel lengths and flat surface at the top of the gels. Before use, the top and bottom of the gels were rinsed with distilled water to remove any unpolymerized gel mixture.

2.5.4.3 Sample loading and IEF separation conditions

The anolyte contained 25 mM phosphoric acid; the catholyte contained 50 mM sodium hydroxide (Duncan & Hershey, 1984). Protein extract (Section 2.5.1)

containing approximately 20 μg protein was loaded on the top of each capillary tube (basic side) and was overlaid with 3 μl of 2D-MH extraction buffer, and then the tube was filled with catholyte. The isoelectric focusing was performed at room temperature with the voltage increased in steps, i.e. 200 V 20 minutes, 400 V 20 minutes and 900 V 20 hours (Mayer *et al.*, 1987).

2.5.4.4 IEF gel removal and storage

Before removing the gels from the tubes, 1 μl of concentrated bromphenol blue comprising 50% (v/v) aqueous glycerol saturated with bromphenol blue was loaded on the top of each gel. The dye marked the basic end of the gel and acted as a tracking dye in the second dimensional gel (Ausubel *et al.*, 1989).

To remove the gels from the tubes, a 5 ml syringe filled with water was connected to the tube via a short piece of rubber tube, and then the gel was slowly forced out by pressure on the syringe. The gels were rinsed with 200 μl transfer solution containing 0.4% SDS and 0.5 M Tris-HCl buffer (pH 6.8) as Burritt (1992) and Hochstrasser *et al.* (1988a) except that no bromphenol blue was added here, then sealed in Eppendorf tubes containing 500 μl transfer solution and immediately stored at -80°C or transferred to SDS-PAGE gels without prior equilibration.

2.5.4.5 Second dimensional SDS-PAGE gels

Uniform 12% T (w/v) gels were prepared as per Smith (1984) using PDA as a cross-linker in place of N,N'-methylene bisacrylamide in order to reduce background staining of the gel matrix during silver staining. The procedure was same as described in Section 2.5.3.2 , but stacking gels were not employed.

2.5.4.6 Protein molecular weight marker preparation

Protein molecular weight marker was prepared according to Ausubel *et al.* (1989). Five microliters of protein molecular weight marker (*BIO-RAD*) was added in 500 μl of 1% agarose in 2× SDS sample buffer (Appendix A-4), mixed and boiled for 5 minutes in a water bath. The hot solution was poured into a glass capillary tube (1.5 mm diameter, *BIO-RAD*). After solidified, the gel was extruded into a clean petri dish and cut into pieces of approximate 0.5 cm, and then stored at -80°C.

2.5.4.7 Loading IEF gel and second-dimensional separation

IEF gels were thawed out for 10-15 minutes at room temperature, and then immediately loaded on the top of the second-dimensional gels. The IEF gels were not sealed with any agarose solution or filter paper and adhered well to the top the second-dimensional gels (Burritt, 1992). Then a piece of the prepared protein molecular weight marker was applied to the basic side of the IEF gel on the top of each second dimensional gel, and the gel was gently overlaid with running buffer. The running buffer was same as before (Appendix 5). Gel electrophoresis was effected at constant 30 mA/gel. No cooling was employed.

2.5.5 Silver staining procedure

Silver staining procedure was basically carried out as per Burritt (1992). At the end of electrophoresis, gels were removed from the glass plates to plastic containers and fixed in 500 ml of fixation solution containing 50% (v/v) methanol and 10% (v/v) acetic acid for 1 hour on an orbital shaker. The gels were then washed 4 times for 5 minutes each with 500 ml of distilled water. Once the gels had been washed they were then soaked individually in 10% (v/v) glutaraldehyde solution for 30 minutes. Extensive washes with distilled water were then performed to remove glutaraldehyde, i.e. 4 times and 15 minutes each followed by an overnight wash. The gels were then stained individually for 15 minutes in an ammonia silver nitrate solution (Appendix A-7). After staining, the gels were washed with distilled water 4 times for 5 minutes each. The proteins were then visualized in a solution containing 0.01% (w/v) citric acid and 0.038% (v/v) formaldehyde (Hochstrasser *et al.*, 1988a). When the image had developed to the required intensity, the development process was stopped by the addition of a solution of 5% (v/v) acetic acid to replace the previous solution. The gels were immediately photographed on Kodak reversal film.

2.5.6 Comparison of 2D gels

Initially, six day 0 gels from 3 separate extractions (2 gels each extraction) were compared, and a base pattern of spots was established, consisting of spots only present in all 3 extractions. Comparison of this base pattern to the patterns observed with

various treatments was carried out in a pairwise-manner (Burritt, 1992). Spots were only deemed to be differentially expressed if the difference was present in at least 2 of the 3 replicate extractions.

2.6 Determination of carbohydrate contents

The sugar and starch contents of cuttings were estimated using a modification of anthrone procedure (McCready *et al.*, 1950) described by Jermyn (1975) and Clegg (1956). In this procedure, a more dilute sulphuric acid solution (75%) was adopted to reduce the rise in temperature (Clegg, 1956) and hydrochloric-formic acid was added before adding anthrone reagent, which considerably enhance the sensitivity and reproducibility of anthrone determination by both increasing the rate of formation of chromogen and reducing the background (Jermyn, 1975).

2.6.1 Extraction of sugar and starch

The method of McCready *et al.*(1950) was adopted. Samples (Section 2.3.1) were ground to a fine powder using a mortar and pestle in liquid nitrogen. Approximately 0.3 g of the fine powder was transferred to a pre-chilled Eppendorf tube, and then 0.3 ml of distilled water was added and vortex mixed. The Eppendorf tubes were kept on ice (to minimize starch degradation by amylase) before 1 ml of hot 80% ethanol was added and vortex mixed for 5 minutes. After centrifugation at 11,000 rpm for 10 minutes, the supernatant was decanted into a new Eppendorf tube. The residue was re-extracted 3 times and the supernatants were pooled. Ethanol interferes with colour development in the anthrone-sugar reaction (Clegg, 1956) and was removed by evaporation in an oven (60°C). The remaining cloudy aqueous fraction was diluted with distilled water to a known volume.

After the final extraction, the residue was suspended in 0.5 ml of distilled water and cooled in an ice bath. The slurry was mixed with 0.65 ml of 52% (v/v) perchloric acid and incubated at room temperature for 15 minutes (vortex occasionally). After incubation, the slurry was centrifuged as before. The supernatant was decanted into a vial and pooled together by three re-extractions as described above. The combined

extract in the vial was diluted to give a final concentration of 25 to 100 μg of starch per ml.

2.6.2 Determination of sugar and starch

In a 25 ml test tube kept on ice the appropriate substances were added in the following order: 0.5 ml of the extract (Section 2.6.1), 0.5 ml of concentrated hydrochloric acid, 50 μl of 90% formic acid and freshly prepared 4 ml of anthrone reagent (0.5 g of anthrone in 500 ml of 75% sulphuric acid solution) which was added slowly enough to avoid excessive frothing. After thorough mixing of the contents, all the tubes, together with others containing appropriate standards and blanks, were heated in a boiling water bath for 12 minutes and immediately cooled in a water bath. Absorbances were read at 630 nm in a Beckman DB spectrophotometer.

To avoid interference by extract and reagent colour, three controls were designed. Control 1 (CT1) consisted of 0.5 ml distilled water, 0.5 ml of concentrated hydrochloric acid, 50 μl of 90% formic acid and 4 ml of anthrone reagent; control 2 (CT2) consisted of 0.5 ml of the extract, 0.5 ml of hydrochloric acid, 50 μl of 90% formic acid and 4 ml of 75% sulphuric acid; and control 3 (CT3) consisted of 0.5 ml of distilled water, 0.5 ml of hydrochloric acid, 50 μl of formic acid and 4 ml of 75% sulphuric acid.

2.6.3 Glucose standards

Two standard curves were prepared by using 0 to 100 μg glucose/ml in water for sugar determination and in a solution containing same amount of perchloric acid as that in the starch extract for starch determination, respectively.

2.6.4 Calculation

The changes in absorbances in the samples were produced by the following equation.

$$\Delta A_{630} = A_{630}(\text{Extract}) - A_{630}(\text{CT1}) - \{A_{630}(\text{CT2}) - A_{630}(\text{CT3})\}$$

The sugar and starch contents were respectively calculated with their own dilution factors being taken into considerations. A factor of 0.9 was used to convert glucose to starch since 0.9 g starch yields approximately 1.0 g of glucose on hydrolysis.

2.7 Enzyme activity

2.7.1 Extraction of crude enzymes

As in protein extraction, samples were collected from the rooting part and ground to a fine powder using a mortar and pestle in liquid nitrogen. Then the powder was transferred to a pre-chilled centrifuge tube and 50 mM phosphate buffer (pH 7.0) was added at a ratio of 1:2 (w:v). The samples were intermittently vortexed for 10 minutes. The samples were then centrifuged at 13,000 rpm (4°C) for 30 minutes. The supernatants were divided into aliquots of 50 µl and stored at -80°C.

2.7.2 Assay of enzyme activity

Three controls were designed to avoid colour interference by substrate and extract. The interference by substrate could be avoided by control 1 (CT1), which was extract blank, i.e. water in place of extract. And the interference by extract could be avoided by the absorbance difference between control 2 (CT2), which was substrate blank, and control 3 (CT3), which was both of extract and substrate blank. The change in absorbance was calculated as follows.

$$\Delta A = A(\text{Extract})-A(\text{CT1})-\{A(\text{CT2})-A(\text{CT3})\}$$

2.7.2.1 Peroxidase

The enzyme extracts were assayed for peroxidase (PO) activity by a modification of the method used by García-Gómez (1995). The enzyme reaction mixture consisted of 50 µl enzyme extract and 1 ml of 0.26 mM *o*-dianisidine and 8.8 mM H_2O_2 in 50 mM Na-phosphate buffer (pH 7.0). CT1 consisted of 50 µl water and 1 ml of 0.26 mM *o*-dianisidine and 8.8 mM H_2O_2 in 50 mM Na-phosphate buffer (pH 7.0); CT2 consisted of 50 µl extract and 1 ml of 50 mM Na-phosphate buffer (pH 7.0); CT3 consisted of 50 µl water and 1 ml of 50 mM Na-phosphate buffer (pH 7.0). The mixtures were

incubated at 25°C for 15 minutes, and changes in absorbance at 460 nm were measured. One unit of the enzymatic activity (U) was defined as the increase of 0.1 absorbance unit per minute under the assay conditions; the enzymatic activity was then expressed in relation to fresh weight (U/min/g F.W.).

2.7.2.2 Polyphenol oxidase

The enzyme extracts were assayed for polyphenol oxidase (PPO) activity by a modification of the method of González *et al.* (1991). The enzyme reaction mixture consisted of 50 µl of enzyme extract and 1 ml of 50 mM pyrogallol in 20 mM Na-phosphate buffer (pH 7.0). CT1, CT2 and CT3 were enzyme extract blank, pyrogallol (substrate) blank and both of enzyme extract and pyrogallol blanks, respectively. The mixtures were incubated at 30°C for 15 minutes, and the changes in absorbance at 420 nm were measured. One unit of the enzymatic activity (U) was defined as the increase of 0.1 absorbance unit per minute under the assay conditions, and the enzymatic activity was then expressed in relation to per gram of fresh weight (U/min/g F.W).

2.7.2.3 IAA oxidase

IAA oxidase (IAA-O) activity was assayed by the method of Huang *et al.* (1977) except that Salkowski reagent was replaced by modified Salkowski reagent (Gordon & Weber, 1951), which may produce a more stable colour and increase specificity and sensitivity. The substrate solution consisted of 0.1 mM 2,4-dichlorophenol (DCP), 0.1 mM $MnCl_2$, and 0.2 mM IAA in 50mM Na-phosphate buffer (pH 6.1) (Huang & Haard, 1977). The enzyme reaction mixture consisted of 50 µl enzyme extract and 1 ml of the substrate solution. CT1 consisted of 50 µl water and 1 ml 50 mM Na-phosphate buffer (pH 6.1); CT2 and CT3 consisted of 50 µl enzyme extract and 50 µl water, respectively, and 1 ml of 50 mM Na-phosphate buffer (pH 6.1).

The reaction mixtures were incubated in a water bath at 37°C for 1 hour and then 2 ml of the modified Salkowski reagent (1 ml of 0.5 M $FeCl_3$, 50 ml of 35% $HClO_4$) (Gordon & Weber, 1951) was added. The mixtures were stored in the dark for 25 minutes and absorbances at 530 nm were determined with a spectrophotometer. Absorbance changes were converted to amount of residual IAA using a curve prepared by plotting the absorbance of standards. The IAA-O activity was defined as

the amount (mg) of IAA destroyed by one-gram fresh tissue per hour under the assay conditions.

2.7.2.4 Amylase

The amylase activity in the enzyme extract was determined by the method described by Bernfeld (1955). The reaction mixture consisted of 50 µl enzyme extract and 1 ml of 1% soluble potato starch in 50 mM Na-phosphate buffer (pH 7.0). CT1, CT2 and CT3 were enzyme extract blank, starch blank and both enzyme extract and starch blanks (i.e. buffer alone), respectively. The mixtures were incubated in a water bath at 30°C for 30 minutes and then the reaction was stopped by adding 0.4 ml of colour development reagent (Bernfeld, 1955) (Appendix A-8). The tubes were boiled in a water bath for 5 minutes, cooled and the absorbances at 540 nm were determined with a spectrophotometer. A standard curve established with maltose (0.1 to 1 mg/ml distilled water) was used to convert the absorbance changes into amount of maltose. The enzymatic activity was defined as maltose produced by one gram of fresh tissue per hour under the assay conditions.

2.7.2.5 Succinic dehydrogenase

Assay of succinic dehydrogenase (SDH) activity of the enzyme extract was based on the methods of Glick & Nayyar (1956) and Defendi & Pearson (1955). The substrate solution consisted of 1 part of 0.1% 2,3,5-triphenol-tetrazolium chloride, 1 part of 0.02 M sodium succinate, 1 part of 50 mM Na-phosphate buffer (pH 7.0) and 1 part of distilled water (Avers, 1958). The enzyme reaction mixture consisted of 50 µl of enzyme extract and 1 ml of the substrate solution; CT1 consisted of 50 µl water and 1 ml of the substrate solution; CT2 consisted of 50 µl enzyme extract and 1 ml of the buffer; and CT3 consisted of 50 µl distilled water and 1 ml of the buffer. The mixtures were incubated in a water bath at 37 °C for 1 hour, then boiled and left at room temperature for 3-4 hours to allow the reduced tetrazolium salt to form precipitate. The red reduced tetrazolium salt by SDH was subsequently collected by centrifugation at 13,500 rpm for 30 minutes, and then dissolved in 1 ml of absolute ethanol. Absorbances at 494 nm were determined with a spectrophotometer.

The absorbance changes were converted into amount of reduced tetrazolium salt by a standard curve, which was prepared with a range of 2,3,5-triphenol-tetrazolium chloride (0 to 1 mg/ml) reduced by excess ascorbic acid. The enzymatic activity was defined as the amount (mg) of tetrazolium salt reduced by one gram of fresh tissue per hour under the assay conditions.

2.8 Histochemistry

2.8.1 Preparation of sections

Free-hand sections (*ca.* 50 μm) were cut from fresh plant material by the aid of a device developed by myself. This device was simply made from a piece of plexiglass (*ca.* 1 cm × 1.5 cm × 5 cm) with a serial holes of different diameters. Hypocotyl was placed in a hole (match the diameters of the hypocotyl and the hole), a razor was used to cut the end of the hypocotyl along the surface of the device. This gave sections with even thickness which were easy to view under a light microscope.

2.8.2 Starch

The sections were stained for starch with an I-KI solution (0.088 g iodine and 4.4176 g KI dissolved in distilled water and made up to 100 ml with distilled water) (Simpson & Naylor, 1962). The sections were then mounted on glass slides and observed under a light microscope.

2.8.3 Enzyme localization

2.8.3.1 Peroxidase (PO)

Sections were transferred into a reaction mixture containing saturated benzidine hydrochloride, 1% (v/v) hydrogen peroxide and 5% (w/v) ammonium chloride at a ratio of 5:5:1 (v:v:v) (van Fleet, 1959; Molnar & LaCroix, 1972b) and incubated at room temperature for 15 minutes. Control was incubated in benzidine hydrochloride

blank reaction mixture. Additional control was a section heat-killed in a boiling water bath for 5 minutes before being incubated in the reaction mixture as above. After staining, sections were mounted on slides and observed under a light microscope.

2.8.3.2 Polyphenol oxidase (PPO)

Sections were transferred into reaction mixture containing 5 mM DOPA in 50 mM Na-phosphate buffer (pH 7.0) (González *et al.*, 1991) and incubated at room temperature for 15 minutes. Control was incubated in 50 mM Na-phosphate buffer (pH 7.0). Additional control was a section heat-killed in a boiling water bath for 5 minutes before being incubated in the reaction mixture as before. The sections were stained and mounted on glass slides and observed under a light microscope.

2.8.3.3 Succinic dehydrogenase (SDH)

The reaction mixture containing 1 part of 0.1% (w/v) neotetrazolium chloride, 1 part of 0.02 M sodium succinate, 1 part of 50 mM Na-phosphate buffer (pH 7.0) and 1 part of distilled water was boiled and cooled before use (Avers, 1958; Molnar & LaCroix, 1972b). Sections were transferred into the reaction mixture and incubated at 37°C for 1 hour. Control was incubated in the above mixture without sodium succinate under the same conditions. Additional control was a section heat-killed in a boiling water bath for 5 minutes before being incubated in the complete reaction mixture. The sections were then mounted on glass slides and observed under a light microscope.

2.8.4 Observation and microphotograph

Photographs of the sections were taken with an Olympus microscope (model BH2-UCD, Japan) equipped with an Olympus C-35AD-4 camera and an Exposure Control Unit on Kodak reversal films.

2.9 Identification of genes expressed during rooting

The strategy of subtractive hybridization was chosen in order to obtain an enrichment of rooting-specific transcripts. The subtracted cDNA was then amplified by PCR and subsequently cloned to a T-vector. A diagram outlining the whole procedure is shown in Figure 2.1.

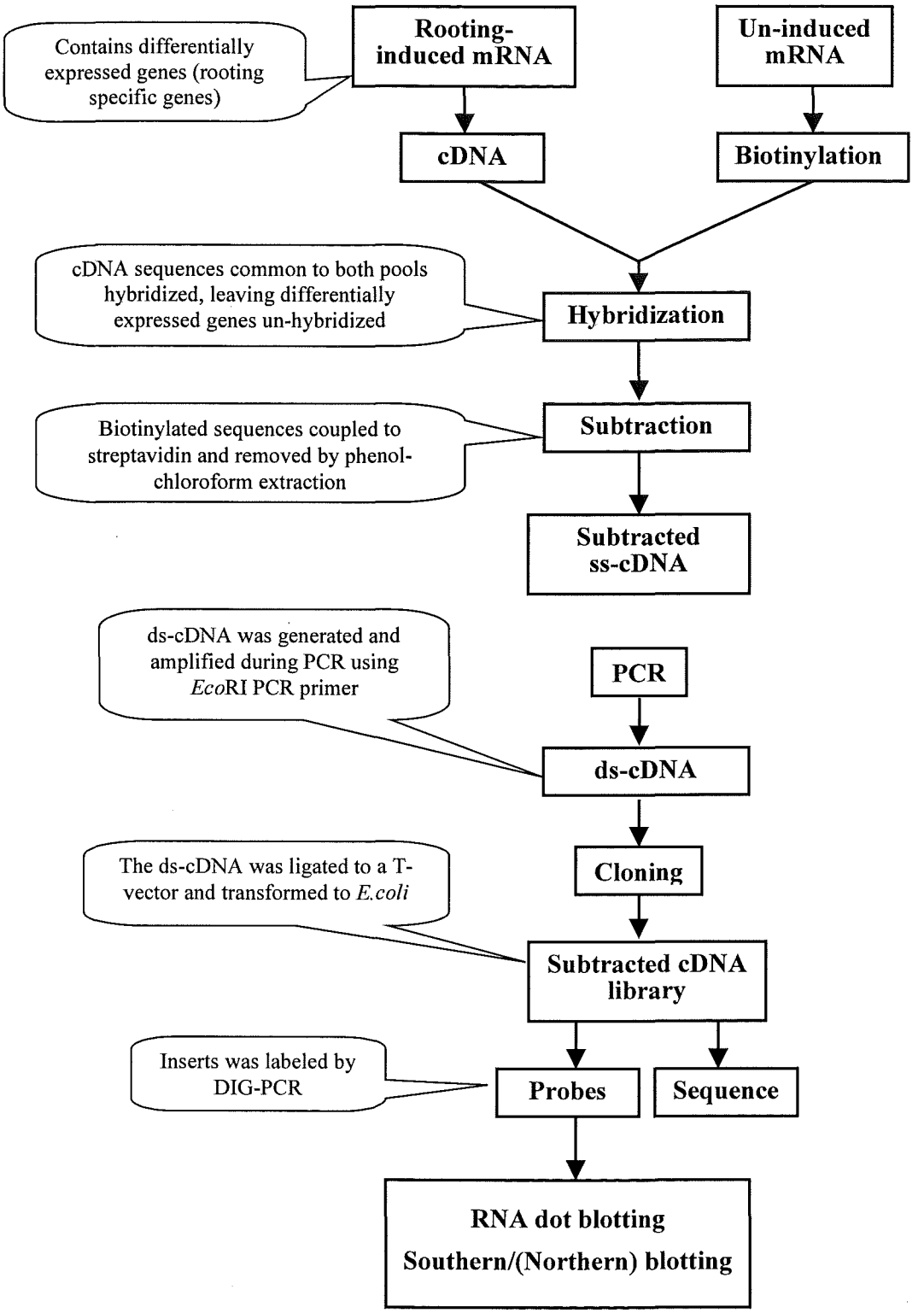

Figure 2.1 Schematic diagram of the molecular cloning and analyses of the genes specifically expressed during adventitious root formation in *Pinus radiata*.

2.9.1 Total RNA isolation

Total RNA was extracted from the rooting region of hypocotyl cuttings treated with or without IBA at day 0, 1, 4, 7, 10 and 13 using TRIZOL® Reagent (*LIFE TECHNOLOGIES*™, Gaithersburg, MD, USA) according to the protocol provided by the supplier. The plant material was ground to a fine powder under liquid nitrogen with a mortar and pestle. About 50-100 mg of the powder was extracted by 1 ml of TRIZOL® Reagent, which is a mono-phasic solution of phenol and guanidine isothiocyanate and an improvement to the single-step RNA isolation method developed by Chomczynski & Sacchi (1987).

2.9.2 Poly(A^+) RNA isolation

Poly(A^+) RNA, namely mRNA, was isolated using an mRNA Isolation Kit (*BOEHRINGER MANNHEIM*) according to the supplier's protocol, starting from total RNA isolated from rooting region of hypocotyl cuttings treated with or without IBA for 7 days, and at the time when cuttings were made (day 0). The principle is that the poly(A^+) tail of mRNA hybridizes to a biotin-labeled oligo(dT) probe, then streptavin-coated magnetic particles capture the biotinylated dT-A hybrids, subsequently the magnetic particles are collected by using a magnetic particle separator and contaminants are removed by washing, finally the mRNAs are eluted from the particles by water.

2.9.3 Generation of subtracted cDNA

To simplify the screening of cDNA library and improve the chances of identifying cDNA clones that correspond to rooting-specific, rare tanscripts, the ss-cDNA synthesized with mRNA from the IBA-induced hypocotyls (day 7) was subtracted with excess poly(A^+) RNA from uninduced hypocotyls (pool of mRNA from hypocotyls at day 0 and uninduced hypocotyls at day 7). The generation of subtracted cDNA was carried out using a Subtractor® Kit (*Invitrogen*®) according to the protocol provided by the manufacturer.

2.9.3.1 Photobiotinylation of uninduced pool of mRNA

To 30 µl containing 10 µg of uninduced mRNA (mRNA from day 0 and day 7 without IBA treatment, 5 µg each), added 30 µl of photobiotin (*Invitrogen®*) solution. The mixture kept in an ice bath exactly 10 cm below a 300W Philips Sunlamp, which produced visible light in the 350-370 nm range, was irradiated for 20 minutes. After adding 50 µl of RNase-free 0.1 M Tris (pH 9.0) (*Invitrogen®*) and 90 µl sterile water, the solution was extracted with 200 µl of water-saturated 2-butanol (*Invitrogen®*) by mixing and centrifugation for 2 minutes. The lower aqueous layer was then mixed with 30 µl of 2 M NaOAc (*Invitrogen®*) and 575 µl of absolute ethanol and frozen at –80°C until a precipitate appeared. The biotinylated mRNA was collected after centrifugation for 10 minutes at 4 °C and washed with 80% (v/v) ethanol. Then the biotinylated mRNA was centrifuged for 5 minutes at 4°C and resuspended in 30 µl of RNase-free water (0.1% (v/v) DEPC treated at 37°C for overnight and then autoclaved).

The mRNA was biotinylated one more time as described above, but this time the extraction with water-saturated 2-butanol was repeated several times until the butanol layer was clear (4-5 times). The biotinylated mRNA was precipitated as above and resuspended in 30 µl of RNase-free water.

2.9.3.2 Synthesis of cDNA from the induced mRNA

About 1 µg of induced mRNA in 16 µl RNase-free water was mixed with 1 µl of oligo dT primer (*Invitrogen®*) and the mixture was incubated at 70°C for 10 minutes. After standing at room temperature for 5 minutes, the following *Invitrogen®* reagents were added to the mixture: 0.5 µl ribonuclease inhibitor, 5 µl 5× RT buffer, 1.25 µl 80 mM sodium pyrophosphate, 1 µl 100 mM dNTP solution and 1 µl reverse transcriptase. The reaction was allowed to take place at 42°C for 60 minutes and was then stopped by adding 2 µl of 0.5 M EDTA (*Invitrogen®*), 2 µl of tRNA (*Invitrogen®*) and 25 µl sterile water on ice. After extraction of the sample with 50 µl of phenol-chloroform (*Invitrogen®*), the cDNA was precipitated by adding 53 µl of 4 M NH_4OAc (*Invitrogen®*) and 265 µl of cold absolute ethanol at –80°C. Then the sample was centrifuged at 16,000 × g at 4°C for 15 minutes and the pellet was resuspended in 100 µl sterile water. The unincorporated dNTPs were removed by the second

precipitation, in which 100 µl of 4 M NH_4OAc and 400 µl of absolute cold ethanol were added to the sample, and the mixture was placed at –80°C until a precipitate appeared. After centrifugation as above, the pellet was resuspended in 50 µl 0.5 N NaOH and incubated at 55°C for 15 minutes to break down the mRNA. Finally, the purified cDNA was precipitated by adding 15 µl of 2 M NaOAc and 230 µl of cold absolute ethanol at –80°C.

2.9.3.3 Subtraction hybridization

Thirty microlitre each of biotinylated uninduced mRNA and induced cDNA were mixed with 9 µl of 2 M NaOAc and 175 µl of cold absolute ethanol so that a precipitate was formed at –80°C. The precipitate was then washed once with 500 µl of ice-cold 80% ethanol, centrifuged (16,000 × g at 4°C for 10 minutes) and the pellet was resuspended in 10 µl of sterile water and 10 µl of 2× hybridization buffer (*Invitrogen*®). For mRNA-cDNA hybridization, the mixture was heated at 100°C for 1 minute, then moved immediately to a 68°C water bath.

Following the hybridization step (48 hours at 68°C), 30 µl of 10 mM HEPES/EDTA buffer (*Invitrogen*®) and 10 µl of streptavidin (*Invitrogen*®) were added to the mixture pre-incubated at 55°C for 5 minutes. The streptavidin was allowed to bind to the biotin in the mixture at room temperature for 10 minutes before being extracted with 60 µl of phenol-chloroform, and then with 50 µl of HEPES/EDTA buffer. The aqueous layers of these two extractions were combined before another 10 µl of streptavidin was added. After incubation at room temperature for 5 minutes, the mixture was extracted again with 120 µl of phenol-chloroform and 50 µl of HEPES/EDTA buffer. To the combined aqueous layers from the two extractions, 25 µl of 2 M NaOAc and 490 µl of ethanol were added to precipitate the subtracted cDNA at –80°C. Here a variation to the protocol provided by the supplier is that tRNA was not added to help the precipitation of the subtracted cDNA as tRNA may interfere with the following step involving terminal transferase (Land *et al.*, 1983). Finally, the subtracted ss-cDNA was recovered by centrifugation (16,000 × g, 4°C, and 15 minutes) and resuspended in 7 µl of sterile water.

2.9.4 Amplification of the subtracted cDNA

After the ss-cDNA was tailed with dATP using terminal transferase, it should contain a poly d(A) tail at 3' end and a poly d(T) tail at 5' end. Then the cDNA was amplified by PCR using a single oligo d(T) containing stablizer sequence (*Eco*RI PCR primer) as primer (Foote *et al.*, 1997), yielding ds-cDNA for further cloning.

2.9.4.1 Tailing of the subtracted ss-cDNA

The subtracted ss-cDNA was tailed at the 3' end with dATP using terminal transferase (TdT). The reaction was performed according to the recommendation of TdT supplier in 20 μl containing 4 μl of 5× reaction buffer, 2 μl of TdT (50 U), 6 μl of 5 mM $CoCl_2$, 1 μl of 1 mM dATP and 7 μl of the cDNA. The reaction was stopped by heating at 65°C for 10 minutes and the tailed cDNA was precipitated by cold 100% ethanol after the reaction mixture was incubated at 37°C for 15 minutes.

2.9.4.2 Generation of ds-cDNA during the amplification by PCR

The tailed ss-cDNA featured a poly d(A) at 3' end and a poly d(T) at 5' end, which was of the oligo d(T) primer used in reverse transcription reaction. This allows the ss-cDNA to be amplified by PCR using *Eco*RI PCR primer, generating ds-cDNA. Optimization of the PCR step was initially set by addition of DMSO, or DTT or Mg^{++} in the PCR buffer (containing 1.5 mM of Mg^{++}, *BOEHRINGER MANNHEIM*). Finally, for PCR amplification of the tailed ss-cDNA, the reaction mixture consisted of 1× PCR buffer, 3.5 mM Mg^{++}, 0.2 mM each dNTP, 1 OD_{260} unit /ml *Eco*RI PCR primer, and 5 units of *Taq* DNA polymerase in a total volume of 50 μl, and a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) was set to run 1 cycle of 94°C × 2 minutes, 40 cycles of 94°C × 30 seconds, 42°C × 30 seconds, 72°C × 3 minutes and a final extension at 72°C × 10 minutes.

2.9.5 Cloning to form the subtracted library

The ds-cDNA generated by PCR was cloned using an Original TA Cloning Kit (*Invitrogen*®) according to the supplier's protocol. The supplied linearized pCR®2.1

vector possesses two single 3' T-overhangs at the two ends. This is designed to clone the PCR product directly without modification of the PCR primer by phosphorylation or addition of a restriction site since the *Taq* DNA polymerase acts on ds-DNA as a non template-directed transferase leaving a single protruding 3' dATP. The pCR® 2.1 also contains *lac Z'* gene and kanamycin/ampicillin resistance genes, which allow easy screening of the recombinants (white colonies) on plates containing X-gal and kanamycin or ampicillin.

2.9.5.1 Ligation

The ligation reaction was performed in 10 µl containing 1 µl, 2 µl or 3 µl of the PCR product, 5 µl of sterile water, 1 µl of 10× ligation buffer (*Invitrogen®*, see Appendix A-12), 2 µl of pCR®2.1 vector (25 ng/µl, *Invitrogen®*), and 1 µl of T4 DNA ligase (4 Weiss units, *Invitrogen®*). The mixture was incubated at 14°C overnight and then placed on ice.

2.9.5.2 Transformation

Two µl of 0.5 M β-mercaptoethanol (*Invitrogen®*) was pipetted into one vial of One Shot™ Competent Cells (INVαF', *Invitrogen®*) thawed on ice and mixed by stirring gently with the pipette tip. Then 2 µl of the ligation reaction was directly added into the competent cells and mixed by stirring gently with the pipette tip. The remaining ligation mixture was stored at –20°C. Following incubation on ice for 30 minutes, the competent cells were heat-shocked for 2 minutes at 42°C and then placed on ice for 2 minutes. After addition of 250 µl of SOC medium (*Invitrogen®*, see Appendix A-13), the vial was shaken horizontally at 37°C for 1 hour at 225 rpm in a rotary shaking incubator. Subsequently, 50 µl and 250 µl of the incubation mixture were spread on LB agar plates (Appendix A-14) containing 50 µg/ml of kanamycin and 40 µl of 40 mg/ml X-gal (Appendix A-15) which were previously spread evenly on the surface of the plates.

The plates were placed at 37°C overnight, and then shifted to 4°C for 2-3 hours to allow for proper colour development.

2.9.6 Identification of recombinant clones

2.9.6.1 Preparation of plasmid DNA and restriction digestion

Plasmid DNA of white colonies was prepared using a small-scale plasmid isolation procedure (Towner, 1991a). Then, approximately 1 μg of the plasmid DNA was digested by 1 unit of *Eco*RI in 20 μl at 37°C for 1 hour. In order to remove contaminating RNA, 0.5 μl of DNA-free RNase A (1 mg/ml) was added to the digestion reaction (Towner, 1991a). The reaction was stopped by adding 6 μl of Type III loading buffer (Maniatis *et al.*, 1982) (Appendix A-16).

2.9.6.2 Electrophoresis of the digested plasmid DNA

Thirty ml of 1% agarose in TAE buffer (Appendix A-17) was poured into a gel mould (6.5 cm × 10 cm) and a sample well-forming comb was immediately placed in position. After polymerization, the comb was removed and the gel was placed in the electrophoresis apparatus (*BIO-RAD, Mini SUB™ DNA CELL*); sufficient buffer was filled in the electrode chambers and the gel was covered with a depth of about 1 mm. Then 10 μl of the sample was loaded into the well. The electrophoresis was performed at 80 V for 1 hour using Model 3000xi Computer Controlled Power Supply (*BIO-RAD*). The gel was viewed using an UV illuminator after staining with 0.5 μg/ml of ethidium bromide for 10 minutes, rinsing in running tap water for 10 minutes.

2.9.7 Probe labelling by PCR-DIG and purification by gel filtration

Two μl of digested plasmid DNA (containing inserts) was used as template to perform the PCR. The conditions and cycle profile were the same as in Section 2.9.4.2 above, except that 2 μl of 1 mM DNA Labelling Mixture (0.65 mM dNTP + 0.35 mM DIG-11-dUTP) and 8 μl of 1 mM dNTP were added to the reaction. The quantification of the labelled PCR product was performed according to the DIG/Genius (tm) User's Manual (*BOEHRINGER MANNHEIM*).

To avoid the interference of free DIG-11-dUTP in the later step of hybridization and detection, the labelled DNA was purified by agarose gel. The labelled DNA was isolated from agarose gel by 'freeze-squeeze' method as described by Towner (1991b), except that a spin-column (*Invitrogen*®) was used other than a punctured microcentrifuge tube.

2.9.8 RNA dot blotting

2.9.8.1 RNA sample treatment

Essentially, the procedure of RNA dot blotting was performed according to the DIG/Genius (tm) User's Manual (*BOEHRINGER MANNHEIM*). Total RNA samples were diluted in RNA dilution buffer (Appendix A-18) to 1 μg/μl. The RNA was fixed to the nylon membrane using an UV transilluminator for 3 minutes after 1 μl of the RNA sample was spotted onto a dry nylon membrane (positively charged, *BOEHRINGER MANNHEIM*).

2.9.8.2 Hybridization

Then the membrane was placed in a vial containing pre-hybridization solution (Appendix A-19) and then incubated at 50°C for 2 hours. After the pre-hybridization solution was discarded, hybridization solution (pre-hybridization solution containing 25 ng/ml heat-denatured probe) was added to the vial. The probe was allowed to hybridize to RNA on the membrane at 50°C overnight. At the end of the hybridization step, the membrane was washed 2 × 15 minutes in 2× wash solution (Appendix A-20) at room temperature, and 2 × 15 minutes in 0.1× wash solution (Appendix A-21) at 68°C.

2.9.8.3 Immunological detection

After hybridization and stringency washes, the membrane was rinsed briefly (1-5 minutes) in washing buffer (Appendix A-22) and was blocked by gently agitating in

block solution (Appendix A-23) for 30 minutes. Then the membrane was incubated in Anti-DIG-AP conjugate solution (Appendix A-24) for 30 minutes. After two washes in washing buffer (Appendix A-22) and equilibration in detection buffer (Appendix A-25) for 2 minutes, the membrane was incubated in colour substrate solution (Appendix A-26) in the dark without shaking overnight. Once the hybridization signals with desired clarity were detected, the membrane was washed with water to stop colour development in the background.

2.9.9 Southern blotting

2.9.9.1 Genomic DNA isolation and restriction enzyme digestion

Total genomic DNA was isolated using the CTAB method as described by Wilkie (1997), and digested by *Bam*HI, *Eco*RI, and *Hind*III. The digested DNA was purified by phenol/chloroform extraction before agarose gel electrophoresis to obtain a better quality of the signal (Leroy *et al.*, 1997).

2.9.9.2 DNA electrophoresis

Approximately15 μg of digested DNA was loaded in a well and electrophoresed in a 0.7% agarose gel.

2.9.9.3 DNA transfer to nylon membrane

After electrophoresis, DNA bands were blotted onto the nylon membrane (positively charged, *BOEHRINGER MANNHEIM*) using TE 70 SEMIPHOR™ SEMI-DRY Transfer Units (HOEFER SCIENTIFIC INSTRUMENTS, San Francisco, California) according to the manufacturer's instructions.

2.9.9.4 Southern hybridization and detection

At the end of transfer, the DNA molecules were fixed on the wet membrane by UV light for 3 minutes. The hybridization and detection procedure were described

previously (Section 2.9.8.2 and 2.9.8.3), except that the hybridization temperature was 40°C.

2.9.9.5 Stripping membranes for re-probing

The procedure for removing colour precipitate and probe from the Southern blots was performed according to the DIG/Genius (tm) User's Manual (*BOEHRINGER MANNHEIM*).

2.10 Root induction by *Agrobacterium rhizogenes*

Various plant tissues were inoculated with two strains of *Agrobacterium rhizogenes* in order to test if this agrobacterium can induce root formation in radiata pine or if it can transform radiata pine tissues.

2.10.1 Bacterial strains

Two wild type strains of *Agrobacterium rhizogenes*, A4T and LBA9402, were used to induce adventitious roots from hypocotyl tissue or adventitious shoots of *P. radiata*. Agropine type strain LBA9402, kindly provided by Dr. David Clapham (Swedish University of Agricultural Sciences, Uppsala), is a rifampicin-resistant derivative of NCPPB1855 (Pomponi *et al.*, 1983) and the binary plasmid p35SGUSINT carries an intron-containing *uidA* gene coding for β-glucuronidase (GUS) (Jefferson *et al.*, 1986) and the gene coding for neomycin phosphotransferase II (NPT II). The bacteria were cultured at 26°C either on solid or in liquid YM medium (*LIFE TECHNOLOGIES*™, Gaithersburg, MD, USA) for 1 to 2 days. When the bacterial suspension was used, its absorbance was adjusted to 0.4-0.5 at 550 nm (Tzfira *et al.*, 1996a).

2.10.2 Co-cultivation

Each of the two strains was applied in five different ways to infect the hypocotyl tissue or adventitious shoots of *P. radiata*.

2.10.2.1 Hypocotyl tissue

Method 1 (M1): hypocotyl segments of *ca.* 1 cm long, two or three from each seedling, were randomly selected and inserted upright into ½ MS medium as described above (Section 2.2.2.1) in petri dishes. The bacteria, grown on solid medium, were inoculated onto the top cut-surface of the segment with a loop, and then the petri dishes were placed in a plant growth room as described above (Section 2.2.1). In order to determine sensitivity of the tissue to auxin, in this method the segments were either transferred to the medium containing 9 mg/l IBA for 1 week at day 7 after inoculation of the bacteria (M1-a) or kept in hormone-free medium during the whole process (M1-b) in the plant growth room. The segments without inoculation with the bacteria were designated as controls.

Method 2 (M2): non-aseptic whole seedlings, raised in vermiculite in small cabinets (22 cm × 15 cm × 12 cm), were used in this method. The hypocotyls were injected with about 10 µl of the bacterial suspension using a syringe fitted with a 24G needle at the site of 1 cm above soil line. Then the cabinets were covered with plastic film and placed in a plant growth room (Section 2.2.1). Control seedlings were treated in the same way except that the bacterial suspension was replaced by liquid YM medium.

Method 3 (M3): de-rooted seedlings were dipped in a bacterial colony grown on solid medium, and then inserted upright into vermiculite saturated with ½ GD (Gresshoff & Doy, 1972) nutrient solution (McAfee *et al.*, 1993) in tissue culture jars (250 ml, clear polycarbonate plastic container from Labserv, Biolab, New Zealand). The segments without inoculation with the bacteria were designated as control. Subsequently, the jars were placed in a plant growth room (Section 2.2.1).

Method 4 (M4): de-rooted seedlings were co-cultivated with a bacterial suspension in tissue culture jars (250 ml, clear polycarbonate plastic container from Labserv, Biolab, New Zealand) for 1 hour and then grown as in M3. Control cuttings were cultured in liquid YM medium for 1 hour.

Method 5 (M5): the same treatment as in M4 except that the co-cultivation period was 24 hours. Control cuttings were cultured in liquid YM medium for 24 hour.

2.10.2.2 Adventitious shoots

Three year old greenhouse-grown seedlings were pruned to promote the development of adventitious shoots which were injected *in vivo* with approximately 20 μl of the bacterial suspension using a syringe fitted a 24G needle at the site of about 10 cm from the tops of the shoots in November 1998. One week later, the shoots were removed from the plants about 0.5 cm below the injection sites, and cultured as described in M2 for 7 weeks. Control shoots were treated in the same way except that the bacterial suspension was replaced by liquid YM medium.

2.10.3 Evaluation of root formation

Survived, rooted seedlings or cuttings, and root number were investigated at week 6 or 8 after inoculation. The morphological characters of the root formation were also observed.

2.11 Statistical analysis of data

All experiments were performed three times. One way analysis of variance (ANOVA) or t-Test were conducted to evaluate the significance of the factors in the study of root induction by *A. rhizogenes*, and of starch content from rooting region of hypocotyls cultured in sucrose-free medium. Data of percentages were either converted by arcsin transformation prior to ANOVA analysis or tested for independence using the Chi-square test. Multiple comparison test was performed using Tukey's method following ANOVA because this method is relatively more conservative, so it is less likely to determine that a given difference is statistically significant and it is the recommended test for all pairwise comparisons. Statistical analysis was performed using the SPSS for Windows statistical software package (SPSS Inc., Version 8.0, 1998).

Chapter 3
Results

3.1 Rooting response

The choice of the plant material was based on a preliminary study showing that the hypocotyl of de-rooted *Pinus radiata* seedling responded and grew well in IBA-containing nutrient medium. Study on different basal media, including MS, ½ MS, 1/5 MS, GD (Gresshoff & Doy, 1972) modified by Reilly *et al.* (Reilly & Washer, 1977), and RIM (Rancillac *et al.*, 1982), different IBA concentrations and treatment times, and different sucrose concentrations was carried out to establish a reliable *in vitro* adventitious rooting system.

3.1.1 The effects of different media on root formation

There was little difference in rooting efficiency (% of rooted cuttings) among different media with the same level of IBA (9 mg/l), particularly GD, ½ MS, ½ MS+ and 1/5 MS, in which rooting efficiency was over 90% (Figure 3.1). The rooting efficiency was 100% in RIM medium, the highest, and 81.0% in MS medium, the lowest. However, the number of roots/cutting was different among these media. Low number of roots/cutting occurred in MS, ½ MS+, 1/5 MS, and GD, 3.38, 3.82, 4.21 and 4.39, respectively. Average 6.45 roots/cutting was recorded in RIM medium. The highest, 8.35 appeared in ½ MS medium. Therefore, this medium had been employed for the bulk of experiments in this thesis.

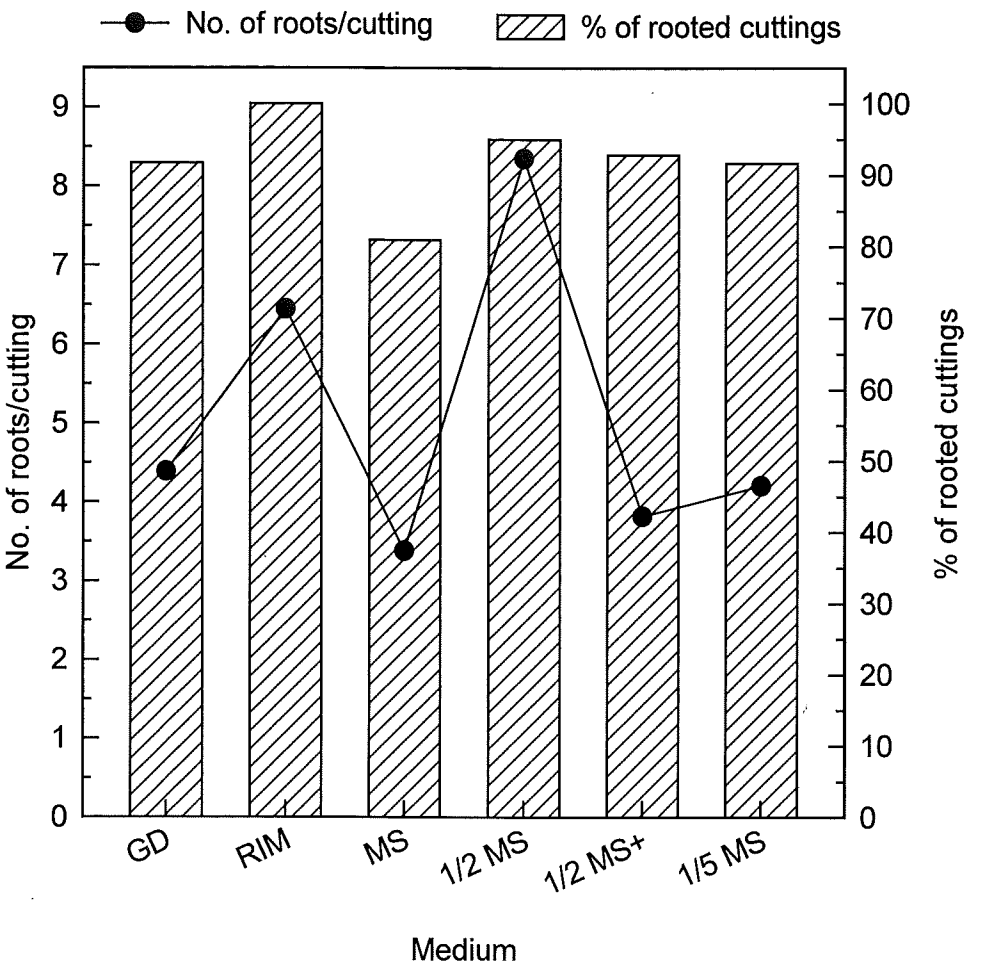

Figure 3.1 The effects of different media on adventitious root formation of *Pinus radiata* (day 30).

3.1.2 The effects of different sucrose concentrations on root formation

Adventitious root formation in de-rooted hypocotyls of radiata pine was dependent on sucrose concentrations in the ½ MS medium supplemented with 9 mg/l IBA (Figure 3.2).

Histological evidence indicated that primordia could be formed in the hypocotyls cultured in sucrose-free medium although the primordium number was much less than that in sucrose containing medium, but most of the primordia failed to further develop into functional roots (observations not shown). This means that the sucrose is not only important to root primordium initiation, but also to primordium development and root growth.

Ten grams per liter sucrose seemed to be not enough for best root formation, while the rooting efficiency and number increased markedly with the increase of sucrose concentrations. It seems that 20 g/l of sucrose is optimum for adventitious rooting in this system. However, there was little difference between the treatments with 20 and 30 g/l of sucrose.

3.1.3 The effects of IBA treatment on adventitious rooting

The effects of different IBA concentrations and exposure times on adventitious root formation were studied (Table 3.1). Overall, it seems that relatively high IBA concentrations and long treatment times were required to efficiently induce adventitious roots in the hypocotyl cuttings of *Pinus radiata*.

Not only IBA concentrations influenced the rooting efficiency and root number per cutting, but also some visible differences at the bases of the hypocotyls of the de-rooted seedlings were exhibited among the different treatments (Plate 3.1). In high IBA concentrations, for instance 9 mg/l, the basal portion of the de-rooted seedlings enlarged substantially from day 7. This was mainly due to more potential meristematic loci involved and the continued cell division of the meristematic tissue, leading to the formation of multiple root primordia. In contrast, significant enlargement of the basal portion of the cuttings could not be observed in the treatment

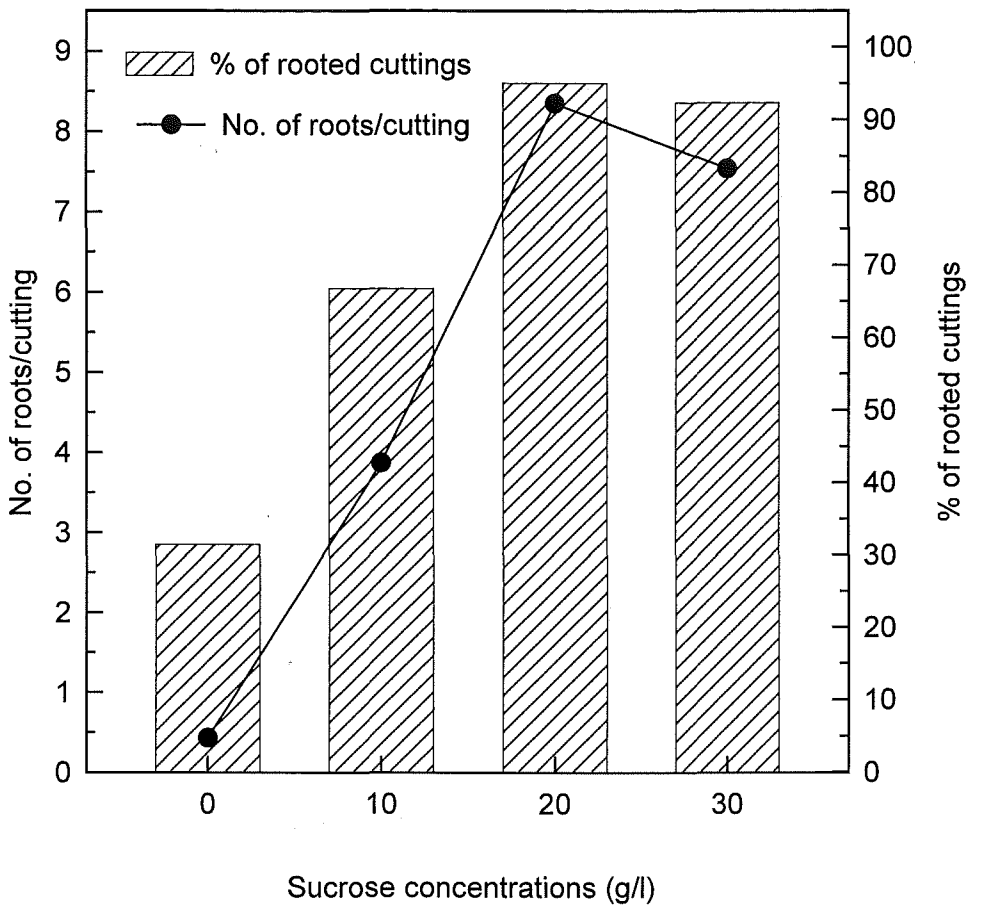

Figure 3.2 The effects of different concentrations of sucrose on adventitious root formation of *Pinus radiata* (day 30)

Table 3.1 The effects of IBA concentrations and treatment times on adventitious root formation in *Pinus radiata*

IBA (mg/l)	Time (days)	No. of cuttings	No. of rooted cuttings	% rooted cuttings	No. of roots	No. of roots /cutting
1	2	21	4	19.0	10	0.48
	4	21	11	52.4	22	1.05
	6	27	20	74.1	43	1.59
	8	30	23	76.7	77	2.57
	10	27	24	88.9	76	2.81
	12	28	25	89.3	79	2.82
	14	30	26	86.7	76	2.53
3	2	27	6	22.2	8	0.30
	4	30	17	56.7	26	0.87
	6	27	20	74.1	55	2.04
	8	30	23	76.7	67	2.23
	10	30	28	93.3	98	3.27
	12	27	25	92.6	97	3.59
	14	30	26	86.7	103	3.43
6	2	29	17	58.6	18	0.62
	4	30	19	63.3	38	1.27
	6	33	28	84.8	83	2.52
	8	29	27	93.1	103	3.55
	10	21	19	90.5	146	6.95
	12	24	23	95.8	127	5.29
	14	27	24	88.9	116	4.30
9	2	21	13	61.9	22	1.05
	4	23	18	78.3	39	1.70
	6	34	26	76.5	76	2.24
	8	30	29	96.7	157	5.23
	10	40	38	95.0	334	8.35
	12	22	19	86.4	158	7.18
	14	28	24	85.7	139	4.96
12	2	30	21	70.0	41	1.37
	4	29	24	82.8	44	1.52
	6	27	23	85.2	68	2.52
	8	21	18	85.7	67	3.19
	10	29	27	93.1	157	5.41
	12	30	26	86.7	132	4.40
	14	29	24	82.8	91	3.14
15	2	29	20	69.0	34	1.17
	4	30	26	86.7	55	1.83
	6	32	22	68.8	85	2.66
	8	30	20	66.7	64	2.13
	10	32	22	68.8	67	2.09
	12	28	18	64.3	58	2.07
	14	34	19	55.9	41	1.21
0(CT)	40	25	2	8.0	2	0.08

* Data were obtained after 30 days from the start of treatments

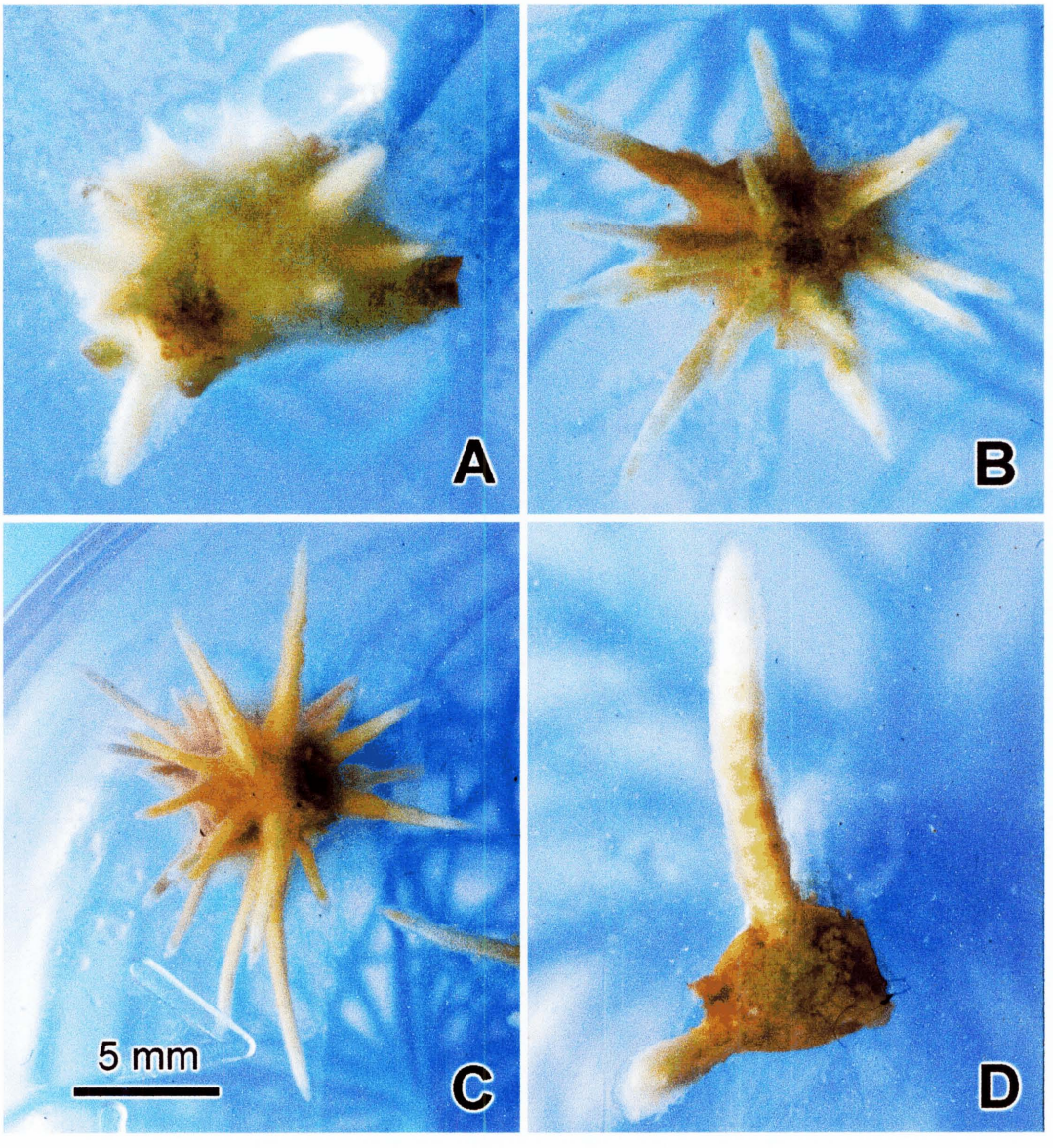

Plate 3.1 Morphology of adventitious root formation in hypocotyls of *Pinus radiata*.

(A) Some roots emerging from the base of a hypocotyl treated with 9 mg/l IBA at day 16. (B) and (C) Further developed roots from the base of hypocotyls treated with 9 mg/l IBA at day 23 and 30, respectively. (D) Three roots with different length from the base of a hypocotyl treated with 1 mg/l IBA.

with low IBA concentrations, particularly 1 mg/l IBA. In control (i.e. no IBA), the morphology of the basal portion was similar to that in the treatment with the low IBA concentration and the appearance of the very few roots was much delayed compared to all the IBA treatments.

3.1.3.1 The effects of IBA concentrations and treatment times on rooting efficiency

In the absence of IBA (CT), Rooting efficiency (% of rooted cuttings) was very poor which increased with increasing IBA concentrations when the treatment time was shorter, i.e. 2 and 4 days (Table 3.1). However, the response to different IBA concentrations became similar when IBA treatment times increased. The best rooting efficiency was observed in the culturing treated with 6 or 9 mg/l IBA for 8 to 10 days.

3.1.3.2 The effects of IBA concentrations and treatment times on number of roots formed

Mean root number per cutting increased in response to higher IBA when the treatment times were from 2-6 days (Table 3.1). The highest root number was achieved in the treatment with 9 mg/l of IBA for 10 days.

In general, it seems that there is a compensatory effect between IBA concentration and treatment time, and the root number was more sensitive to IBA concentration and treatment time than rooting efficiency was.

3.1.4 The effect of kinetin on adventitious rooting

Adventitious root formation was strongly inhibited by kinetin, both 10 mg/l of kinetin combined with 9 mg/l of IBA and the kinetin alone (data not shown). No roots were observed with these two treatments at day 30, but some visible differences were evident. As in control (i.e. no IBA), enlarged basal portion of the hypocotyl was not observed in treatment with kinetin alone. However, in the treatment of kinetin combined with IBA, enlarged basal portion was observed, apparently due to proliferation of callus instead of root primordium differentiation and development.

3.2 Developmental sequence of root formation

The main aim of this section is to investigate the relationship between stages of root formation and exposure time to 9 mg/l IBA in the culture medium (see Section 3.1.1) in order to establish sampling times for various analyses. The present investigation indicated that the developmental sequence of adventitious root formation in hypocotyls of *Pinus radiata* in the rooting system here is very similar to that described by Smith & Thorpe (1975a), consisting of three distinct phases.

3.2.1 Pre-initiation phase

At the beginning of culture, the hypocotyl of *Pinus radiata* was frequently seen to have six arms of protoxylem other than five as mentioned by Smith & Thorpe (1975a). This is probably due to different sources of the seeds. In this stage (between day 0 to 3), no histological changes had been observed in the hypocotyl sections from the start of culture (Plate 3.2-A).

3.2.2 Initiation phase

First signs of initiation, including the enlargement of nuclei and the prominence of nucleoli, were observed in the cell at the margin of a differentiating resin duct or in parenchyma tissues external to this tissue at day 4 although this phenomenon occasionally appeared at day 3 (Plate 3.2-B). Following these changes, these cells began to divide. Visible chromosomes were frequently observed at this stage (Plate 3.2-B and C). According to Smith & Thorpe (1975a), this region is referred to as a 'meristematic locus'. Continued division of these cells led to the formation of spheres of meristematic tissue at day 7 (Plate 3.2-D and E). Root primordia were formed by the subsequent division of the meristematic derivatives and ready to emerge through cortical tissue and epidermis at day 10 (Plate 3.2-G).

In addition, two meristematic loci could be initiated and developed simultaneously in the same region (Plate 3.2-E and F), which was located at the margin of the differentiating resin ducts or in parenchyma tissue external to this tissue.

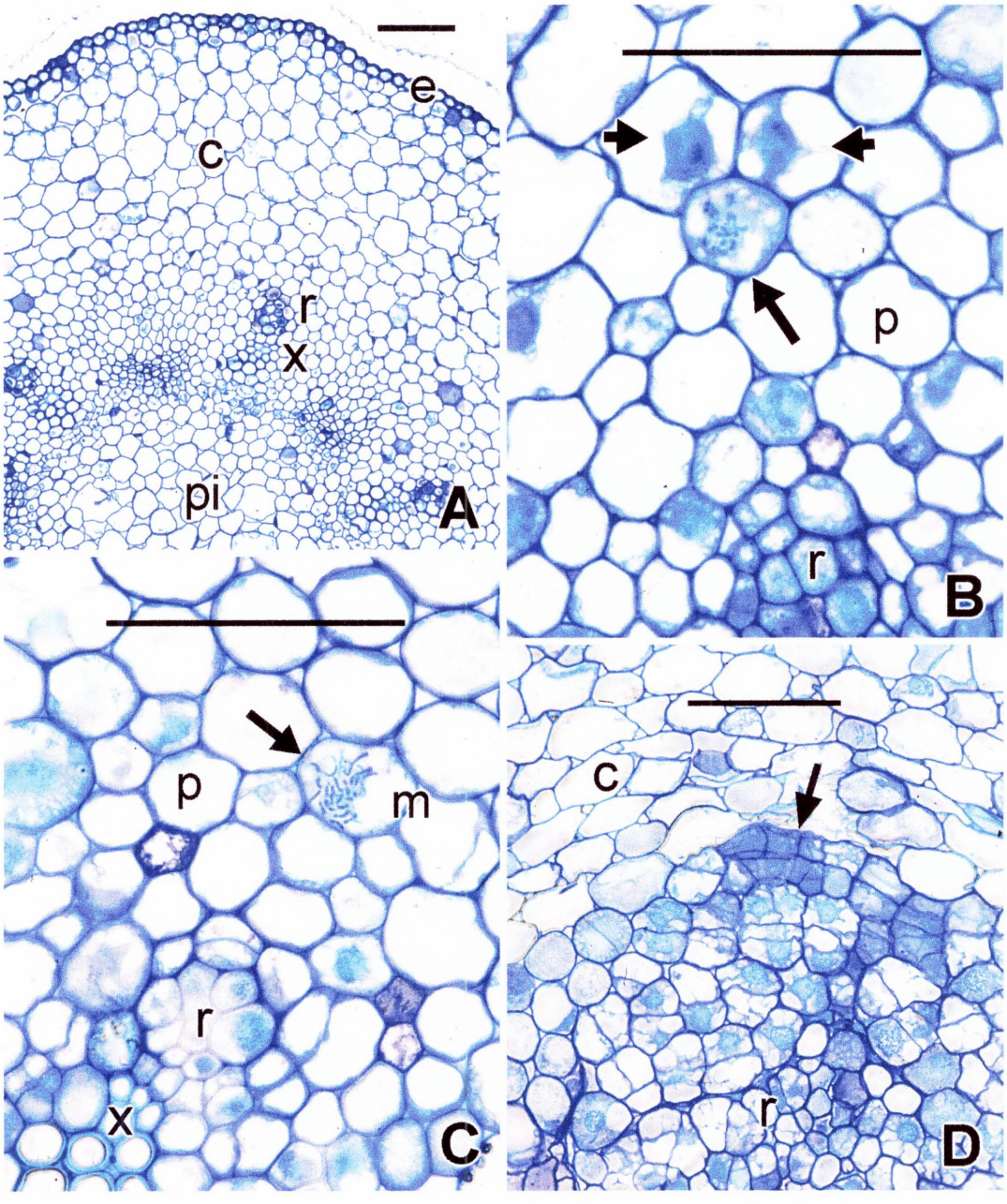

Plate 3.2 Transverse sections of hypocotyls in *Pinus radiata* during adventitious root formation.

m = Meristematic locus, c = Cortex, p = Peripheral cell, r = Differentiating resin duct, x = Protoxylem, e = Epidermis, pi = Pith. All bars = 100 μm. (A) At the time when cuttings were made (day 0). (B) Cells with enlarged nuclei and prominent nucleoli (arrowheads) and a cell with chromosomes (arrow) at day 4. (C) A dividing cell with chromosomes during late prophase or metaphase at day 4 (arrow). (D) Meristematic tissue at day 7 (arrow).

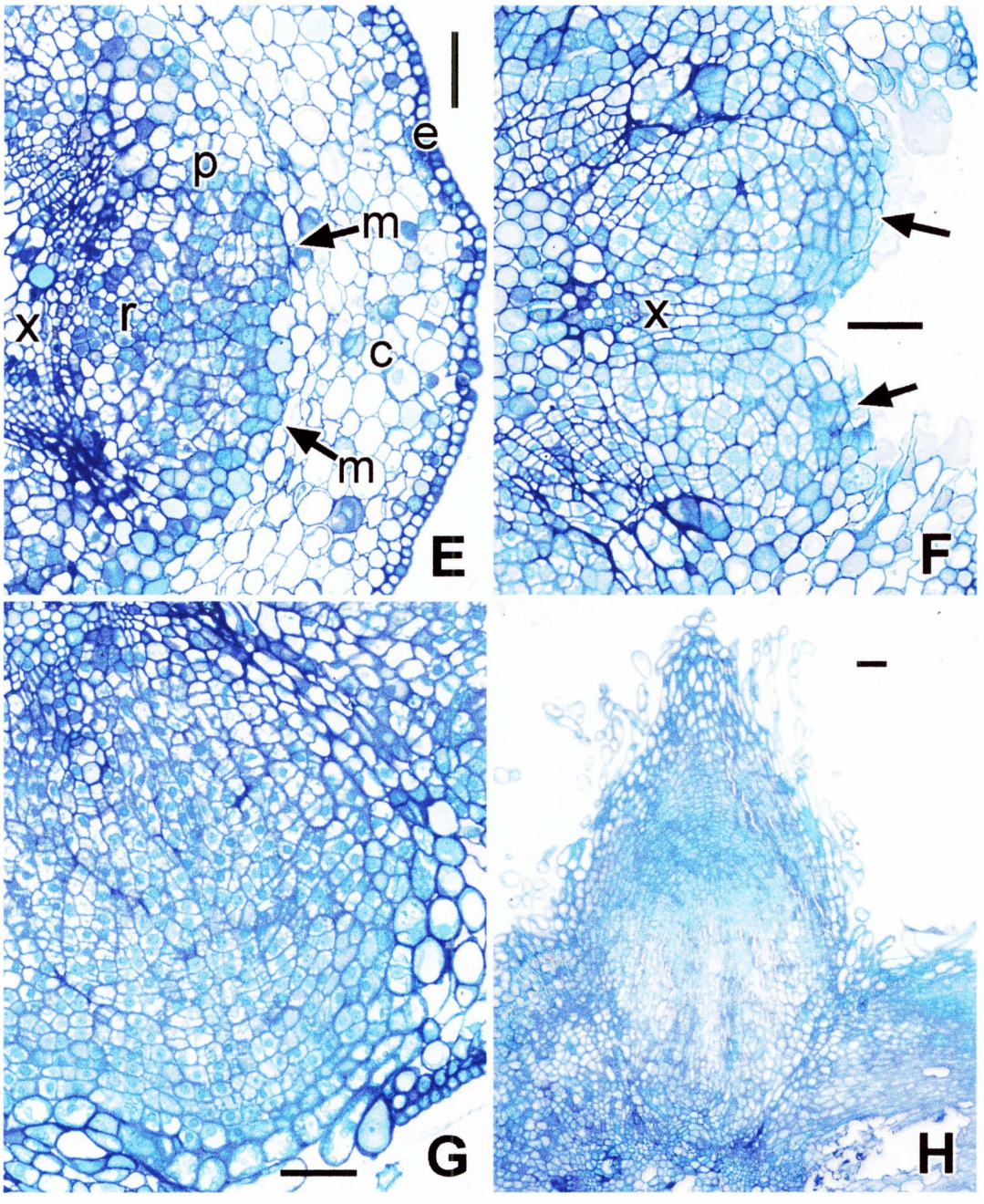

Plate 3.2 (continued) Transverse sections of hypocotyls in *Pinus radiata* during adventitious root formation.

m = Meristematic locus, c = Cortex, p = Peripheral cell, r = Differentiating resin duct, x = Protoxylem, e = Epidermis. All bars = 100 μm. (E) Two groups of meristematic tissue at day 7 (arrows). (F) Two groups of meristematic tissue at day 9 (arrows). (G) A root primordium at day 10. (H) An emerged full differentiated adventitious root at day 13.

3.2.3 Post-initiation phase

At this stage (after 10 days in culture), the primordia developed further and fully differentiated adventitious roots emerged from the basal part of the hypocotyls at about day 13 (Plate 3.2-H).

3.3 Change of fresh weight

The fresh weight of the cut end of the hypocotyl increased slightly in the kinetin treatment and control (i.e. growth regulator-free medium) during the entire time course. Similarly, in the IBA and IBA+kinetin treatments, the fresh weights gradually increased until day 7. Then they sharply increased in the 2 IBA-based treatments (Figure 3.3).

3.4 Protein changes

3.4.1 Protein content

An analysis of the amount of buffer (the same buffer used for 1D SDS-PAGE) soluble proteins showed that the protein content dropped in all the treatments during the first day (Figure 3.4). Then the protein contents in the rooting region of the hypocotyls cultured in the kinetin-containing media with or without IBA dropped markedly from day 4 onwards. In the other non-root inducing control, i.e. the growth regulator-free medium, the soluble protein content remained the same between day1 and day 7, but then it also started to decline to a lower level. In contrast, under the root-inducing condition (i.e. the IBA treatment), there was a gradual increase in soluble protein content till day 7 before it started to decrease.

3.4.2 Protein changes shown by 1D SDS-PAGE

Basically, changes of protein pattern in different hormone treatments at the same time were not observed (Plate 3.3 and Plate 3.4). However, it is interesting to note that there was a day 0 specific protein (◆) which disappeared after the excision of hypocotyls in all cases, while there were two proteins (◀) which seemed to increase

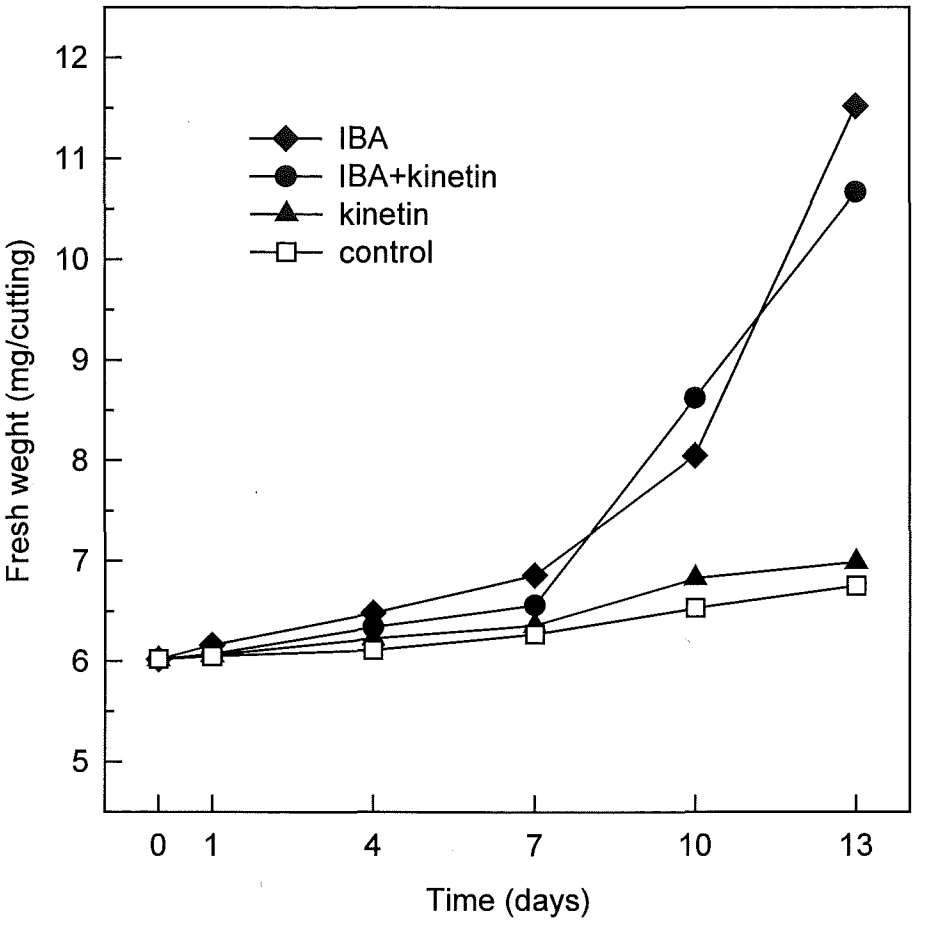

Figure 3.3 Fresh weight changes in the hypocotyl rooting region of *Pinus radiata* during rooting.

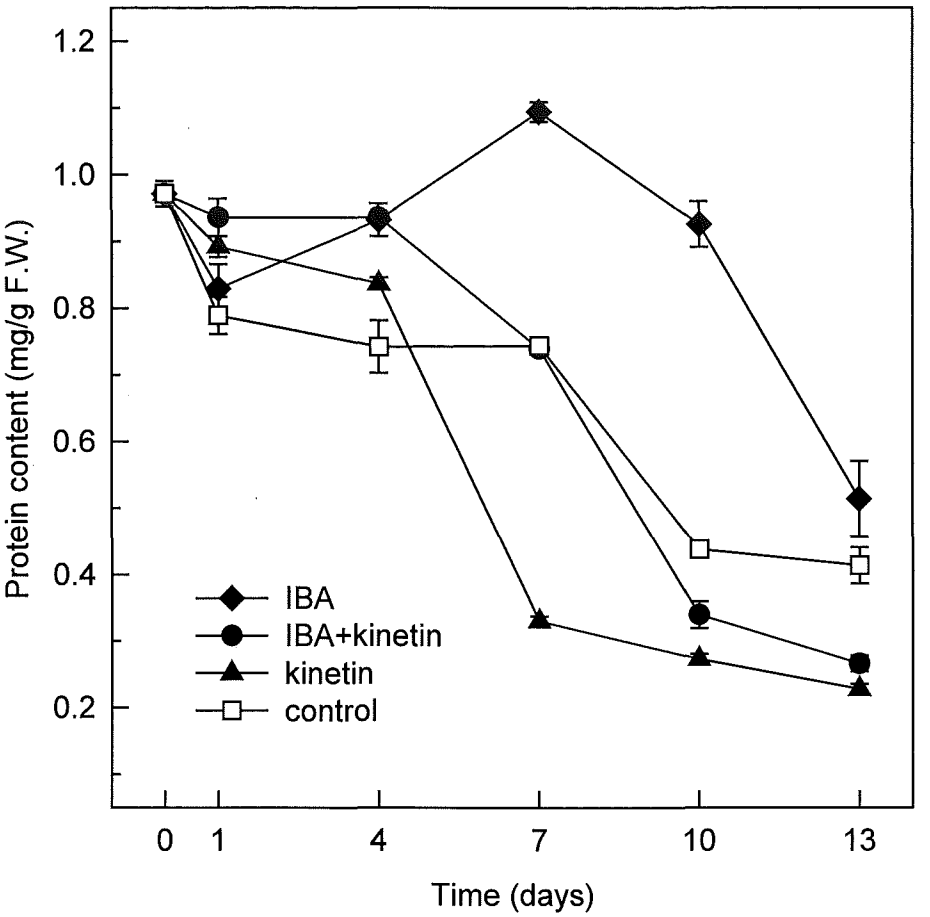

Figure 3.4 Changes of protein content in the hypocotyl rooting region of *Pinus radiata* during rooting.

Vertical bars represent mean ± SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

in abundance as a response to wounding and/or culturing. Several proteins (←) decreased and/or disappeared during culture. One dimensional SDS-PAGE may be not suitable to detect subtle changes in protein patterns during adventitious root formation in some species, as it is likely that only few cells might be involved in root initiation.

3.4.3 Changes of protein patterns shown by 2D PAGE

In order to gain a better insight into changes in gene expression associated with adventitious root formation in hypocotyls of *Pinus radiata*, comparisons between the patterns of protein extracted from hypocotyl cuttings treated with IBA (rooting) and without phytohormones (control) at particular times were made following 2D-PAGE. Five hundred and forty-two protein spots were observed at day 0 when the cuttings were taken (Plate 3.5) and this pattern was considered the control pattern to which all other patterns were compared. Preliminary comparison indicated that major changes of the protein patterns were located in the region below 45 kDa in molecular weight. In addition, the resolution of proteins of higher molecular weights on 2D-PAGE is relatively poor (Dhindsa *et al.*, 1987). Therefore, particular attention was paid to the region below 45 kDa, and 290 proteins detected at day 0 in this region were carefully compared to other treatments. In this way, 46 proteins were found to be newly synthesized in treatments with IBA and/or control during *in vitro* culture.

The changes of protein patterns showed a varied spectrum and the proteins were divided into 7 major groups with several subgroups (Table 3.2). The behaviour of individual protein was listed in Appendix B.

Group 1 includes 108 of the 336 proteins and is designated as the products of basic metabolism or "housekeeping gene" products. These proteins were detected at an approximately constant level throughout the time course in both IBA treatment and control (Table 3.2 and Appendix B). In this group, 24 proteins appeared to be present in relatively large quantities.

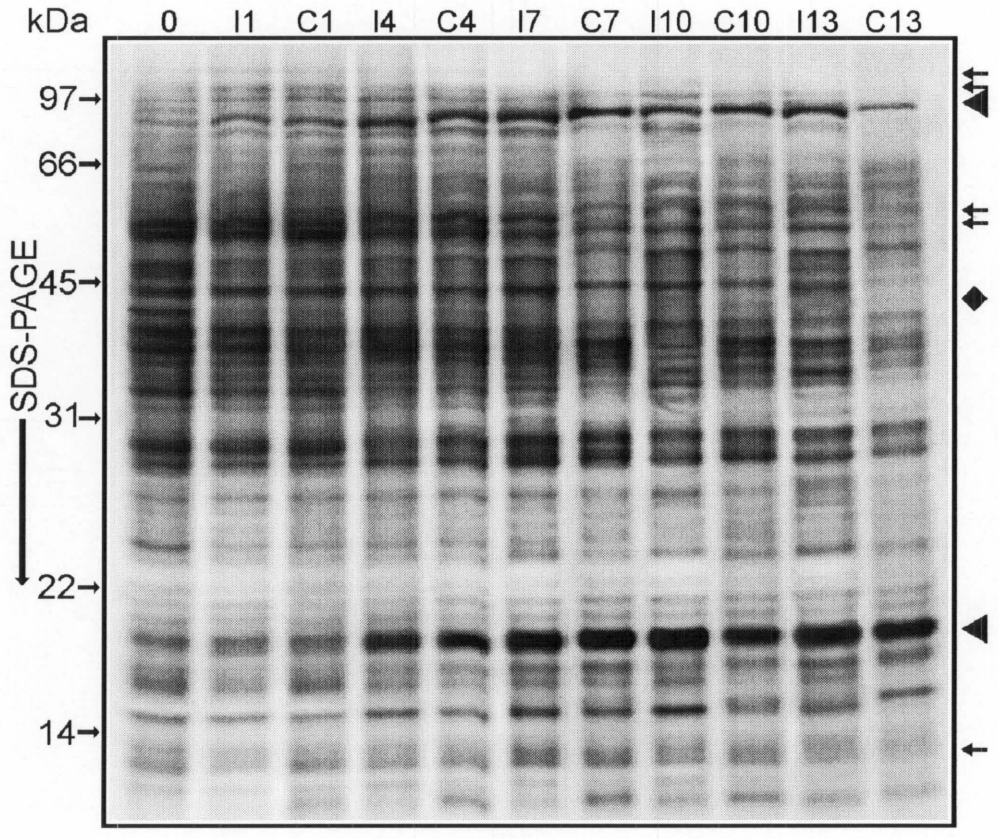

Plate 3.3 Silver-stained SDS-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA (I), control (C) at day 0, 1, 4, 7, 10 and 13.

The numbers on the left indicate molecular weight in kDa. The numbers following letters on the top indicate the treatment time. Some changes of interests are indicated as follows: a protein specific to day 0 (◆); changes in response to wounding and/or *in vitro* conditions (◀: increase; ←: decrease in staining intensity).

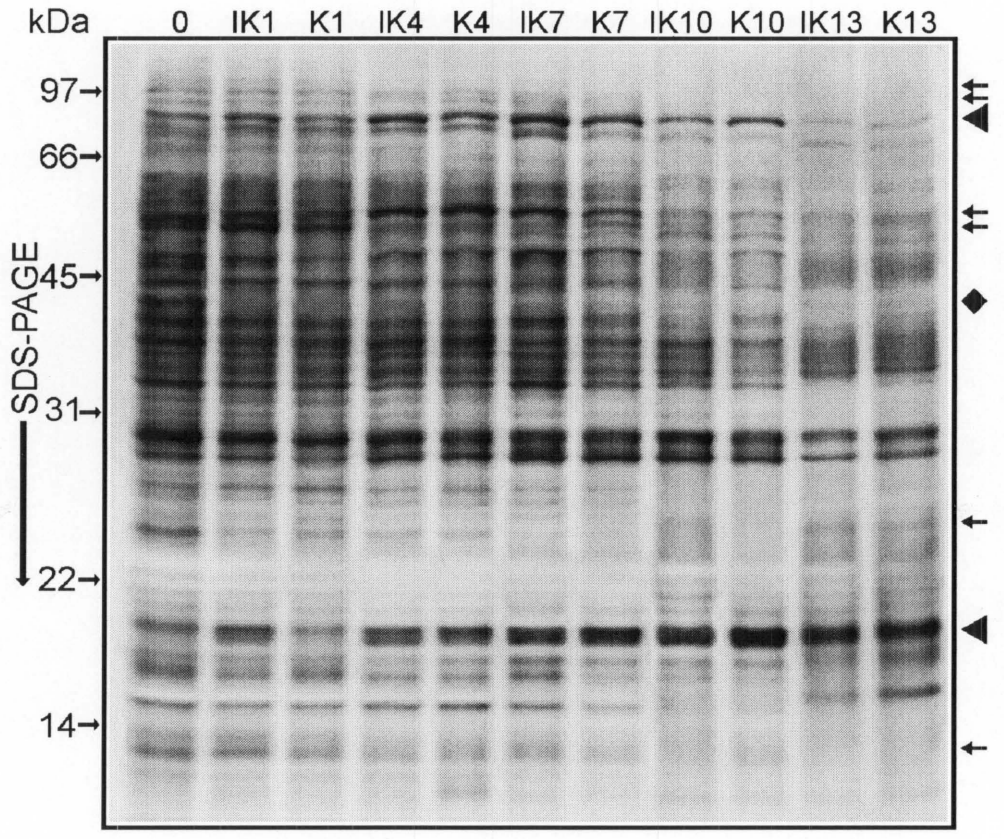

Plate 3.4 Silver-stained SDS-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA+kinetin (IK) and kinetin alone (K) at day 0, 1, 4, 7, 10 and 13.

The numbers on the left indicate molecular weight in kDa. The numbers following letters on the top indicate the treatment time. Some changes of interests are indicated as follows: a protein specific to day 0 (◆); changes in response to wounding and/or *in vitro* conditions (◀: increase; ←: decrease in staining intensity).

Group 2 including 134 proteins may be related to hypocotyl physiology and/or autotrophic growth because they disappeared or decreased upon culture in both IBA treatment and control during the time course.

The proteins in Subgroup 1 were day 0 specific proteins. There are 15 proteins in this category as they disappeared after the excision of hypocotyls (Plate 3.5, marked in ✧). This may be one reason why protein content dropped at day 1 in all cases as shown in Fig. 4. Dhindsa *et al.* (1987) also found that some protein spots considerably decreased or disappeared during 24 hr treatment with auxin, anti-auxin and hormone-free control.

The proteins in Subgroup 2 disappeared in both IBA treatment and control at the same time. There are 11 proteins in this subgroup, in which 5 proteins (No. 20, 35, 48, 80, and 81) were present at day 1 and then disappeared thereafter; 3 proteins (No. 77, 186, and 257) were present till day 4 and then disappeared; No. 111 and 94 were present till day 7 and day 10, respectively, and then disappeared; and No. 215 disappeared from day 7 to day 10, but appeared again at day 13.

In Subgroup 3, there are 30 proteins which disappeared from control. The IBA treatment seemed to retard their disappearance.

In Subgroup 4, there are 18 proteins that were initially absent from the IBA treatment. In contrast to subgroup 3, it seems that IBA could accelerate the disappearance of these proteins.

Subgroup 5. These proteins disappeared at some stage(s) during culture. There are 60 proteins in this subgroup. The roles of these proteins are unclear.

Group 3 comprises 34 proteins that were present at day 0 and control, but disappeared from IBA treatment only. The disappearance of these proteins may be due to some unknown roles played by IBA directly or indirectly. There are 5 proteins (No. 11, 17, 34, 36, and 70) which were more abundant at the beginning of the process in both IBA treatment and control, but then decreased in control and disappeared from IBA treatment.

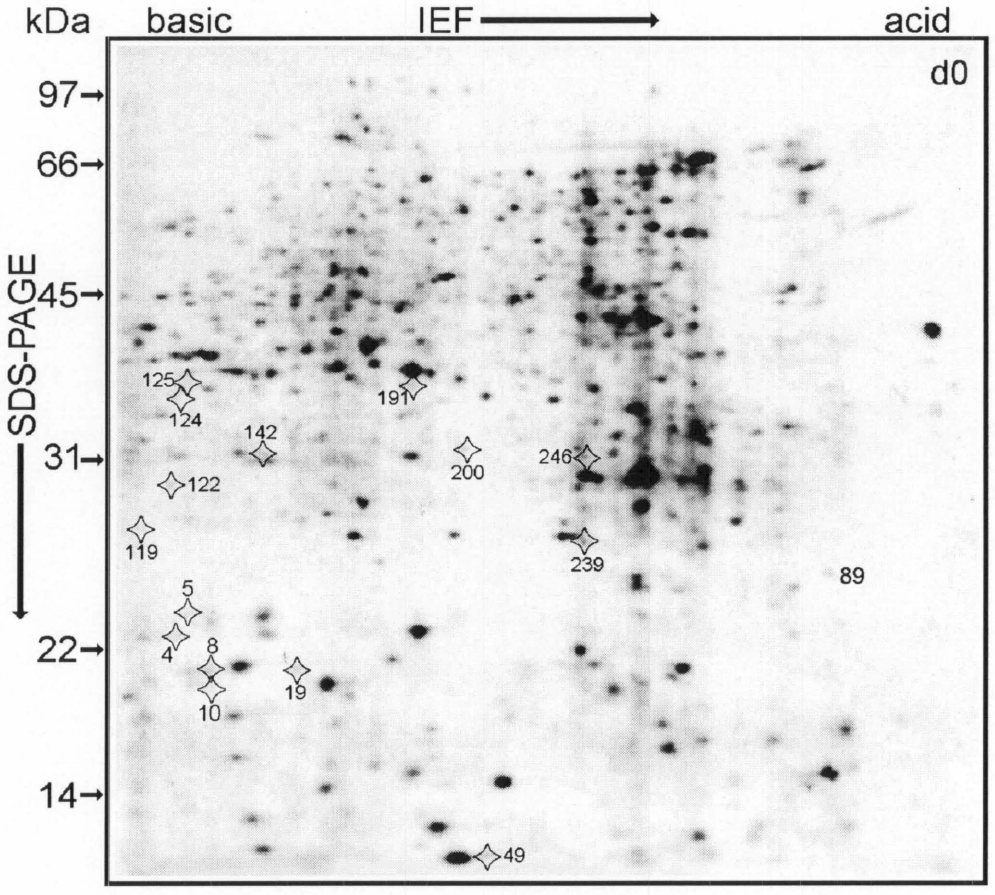

Plate 3.5 Silver-stained 2D-PAGE of proteins extracted from hypocotyls of *Pinus radiata* when the cuttings were made (day 0).

Proteins which appeared at day 0 but disappeared thereafter (in ✧) are indicated.

Table 3.2 Summary of changes in the patterns of proteins associated with IBA and without IBA (control) treatments

1 Basic metabolism (total 108, 32.1%)

These proteins were detected at an approximately constant level throughout the time course in both IBA and control and was presumed to represent the products of basic metabolism or housekeeping gene products

15, **21**, 24, 28, **29**, 30-32, 45, 46, **47**, **52**, **58**, 59, 62, 63, 66, 69, 72, 73, **78**, 82, 83, 91, 92, **113**, 126, **127**, 128, 129, 132-136, 144, 145, 147-150, 153, 154, **156**, **161**, 163, 164, 167, 171, **173**, **175**, 176, **177**, 182, **183**, 187, **188**, 189, 190, **192**, 193, 199, 216, **218**, 221, 224, 233, **234**, 235-237, 241, 242, **243**, 244, 245, 248-250, 253, 256, 258, **262**, **263**, 265, 266, **267**, 268, 269, 275, **276**, 279, 283-285, 290-292, 294-297, 299, 303-306, **321**

2 Disappeared or decreased in both IBA treatment and control (total 134, 39.9%)

These proteins may be related to hypocotyl physiology and/or autotrophic growth

2.1 day 0 specific proteins (total 15, 4.5%, marked in ✧)

[4], [5], 8, 10, 19, 49, [119], 122, 124, 125, 142, 191, 200, 239, 246

2.2 disappeared from both IBA treatment and control at the same time (total 11, 3.3%)

20, 35, 48, 77, 80, 81, 94, 111, 186, 215, 257

2.3 disappeared firstly from control (total 30, 8.9%)

[1], 23, 33, 44, 50, 56, 67, 71, 86, 89(▲), 95, 112, 115, 120, 121, 130, 131, 137, 165, 172, 179, 204, 225, 231, 254, 259, 271, 313-315

2.4 disappeared firstly from IBA treatment (total 18, 5.4%)

[2], 18, 26, 60, 61, 85, 88, 93, 139, 146, 162, 168, 185, 247, 261, 270, 273, 298

2.5 disappeared irregularly at some stage(s) during the culture (total 60, 17.9%)

[3], 7, 9, 14, 25, 27, [37], 51, 54, 55, 57, 87, 90, 96, [116], [117], 123, 138, 140, 141, 143, 158, 159, 166, 169, 180, 181, 194-197, 198, 219, 220, 223, 226-228, 230, 232, 251, 260, 272, 277, 278, 282, 300, 301, 307, 308, 310-312, 316, 317-320, 323, 324

3 Present at day 0 but disappeared from IBA only (total 34, 10.1%)

The proteins could be inhibited by IBA or be related to shoot growth

6, **11**(-), 12, 16, **17**(-), **34**(-), **36**(-), 53, 65, 68, **70**(-), 74, 76, 79, 84, [110], [114], 152, 155, 160, 170, 217, 238, 255, 264, 280, 281, 286-289, 293, 302, 309

4 Presented at day 0 but disappeared in control only (total 14, 4.2%)

IBA may be required to maintain the proteins after excision or the proteins may be related to original root growth

13, 22, 64, [118], 151, 157, [174], 184, 222, 229, 240, 252, 274, 322

5 Newly appeared in both IBA and control (total 21, 6.3%)

Due to metabolism changes, wounding or/and tissue culture

5.1 appeared in IBA treatment and control at different time (total 9, 2.7%)

97, 100, 102, [201], 206, 208, 210, 214, 330

5.2 appeared in both IBA treatment and control at the same time (total 12, 3.6%, marked in □)

39(+), **40**(+), 99, **101**(+), 178, 205, 207, 209, 325, 327(▲), 331, 332

6 Newly appeared in control only (total 6, 1.8%, ≻ indicates)

This may be related to hypocotyl growth, but be inhibited by IBA in the treatment with IBA

75, 98, 106, 107, 108, 109

7 Newly appeared in IBA only (total 19, 5.7%)

7.1 induced by IBA and may be not related to root formation because appeared at day 1 thereafter (total 4, 1.2%, marked in △)

38, [202], [203], 326

7.2 associated with root initiation and formation (day 4-7) (total 4, 1.2%, marked in ◇)

42, 328, 329, 333

7.3 associated with root formation and development (day 7-13) (total 7, 2.1%, marked in ○)

104, 105, 211, 212, 213, 334, 335(+)

7.4 associated with root development only (day 10-13) (total 2, 0.6%, ≺ indicates)

43, 336

7.5 associated with whole process of root formation (day 4-13) (total 2, 0.6%, → indicates)

41 and 103(▲)

Bold large abundance at beginning and/or later (if applicable)
[ ] may not be reliable due to at the basic end
(+) increased in abundance according to the time sequence
(-) decreased in abundance according to the time sequence
(▲) increased firstly and then decreased

Group 4 includes 14 proteins that were present at day 0 and in IBA treatment, but disappeared from control. IBA may be required to maintain the proteins after excision or the proteins may be related to original root growth.

Group 5 comprises 21 proteins that appeared in both IBA treatment and control after excision of hypocotyls. These proteins were considered as a response to wounding and/or tissue culture. Of the 21 proteins, 9 appeared in IBA treatment and control at different times, while 12 appeared in both IBA treatment and control at the same time during the time course from day 1-13 (Plate 3.6 - Plate 3.15, marked in □). In particular four of them numbered 39, 40, 101 and 327 were firstly detectable at day 1-4, then increased markedly to their maximum levels at day 4-7, and subsequently remained relatively constant (No. 39, 40 and 101) or declined a little (No. 327). This can be matched to the band in 1D SDS-PAGE (Plate 3.3 and Plate 3.4).

Group 6 includes 6 proteins (75, 98, 106, 107, 108 and 109) which appeared in control only (Plate 3.7, Plate 3.9, Plate 3.11, Plate 3.13 and Plate 3.15). Number 75 appeared from day 10 to 13; No.107, 108 and 109 were present from day 4 to 7; and number 98 and 106 were detected during the whole process of rooting (day 1 to 13). These proteins may be related to hypocotyl and/or shoot growth, but be inhibited by IBA in the treatment with IBA.

Group 7 comprises 19 proteins which were associated with IBA treatment (rooting) and which are of great interest in the present study. This group was divided into 5 subgroups according to the timing of their appearance.

Subgroup 1 including 4 proteins (No. 38, 202, 203 and 326) which appeared since day 1 (Plate 3.6, Plate 3.8, Plate 3.10 and Plate 3.12, marked in △). Number 202 and 326 appeared from day 1 to 7; number 203 was present from day 1 to 10; number 38 appeared at day 1, 4 and 10, but it could not be detected at day 7. These proteins were probably induced by IBA as histological study indicated that the first cell division began at day 4. It is worth to note that these proteins are located at the basic region.

Subgroup 2 comprises 4 proteins (No. 42, 328, 329 and 333) as well, which appeared during day 4-7 (Plate 3.8 and Plate 3.10, marked in ◇). Number 42 and 333

were detected at day 7, while number 328 and 329 appeared from day 4 to 7. These proteins may be specific to root primordium initiation.

Subgroup 3 includes 7 proteins (No. 104, 105, 211, 212, 213, 334 and 335) which appeared during day 7-13 (Plate 3.10, Plate 3.12 and Plate 3.14, marked in ○). Number 213 and 334 appeared at day 7 and 10, while the other 5 proteins were present from day 7 to 13. This subgroup was designated as an association with root primordium formation and root development.

Subgroup 4 comprises 2 proteins (No. 43 and 336) which appeared during day 10-13 (Plate 3.12 and Plate 3.14, indicated by ➤). Number 43 appeared from day 10 to 13, while number 336 was present at day 10 only. They may be associated with root development only. Some of these proteins in Subgroup 3 and 4 may be root-specific proteins and could be detected in root tissue (original and adventitious roots), particularly the protein numbered 335 in subgroup 3 increased in abundance according to the time course.

Subgroup 5 includes 2 proteins (41 and 103) which were present during the whole rooting process, i.e. day 4-13 (Plate 3.8, Plate 3.10, Plate 3.12 and Plate 3.14, indicated by →). One of the two proteins, numbered 103, was particularly interesting as it appeared firstly at day 4, then increased in abundance till its maximum at day 7 and subsequently decreased.

Very similar to the situation of Oliver *et al.* (1994), many of the changes in proteins were of such small magnitude that it was difficult to quantify them and establish them as novel proteins although many proteins may appear to be associated with root formation. The technique of 2D-PAGE seems to be limited in its ability to consistently track such small alterations. Therefore, a more powerful tool, such as a molecular technique, could be used to detect small changes in gene expression associated with root formation.

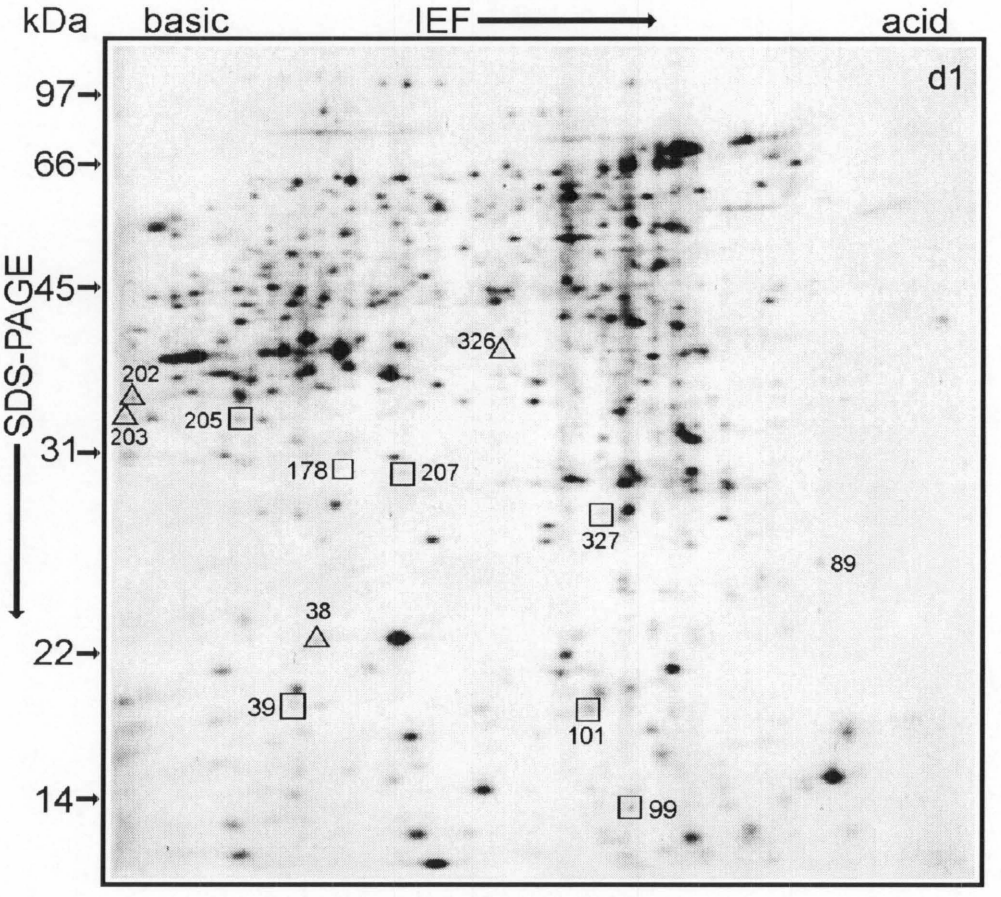

Plate 3.6 Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA at day 1.

Proteins associated with wounding (in □), induced by IBA (in △) are indicated.

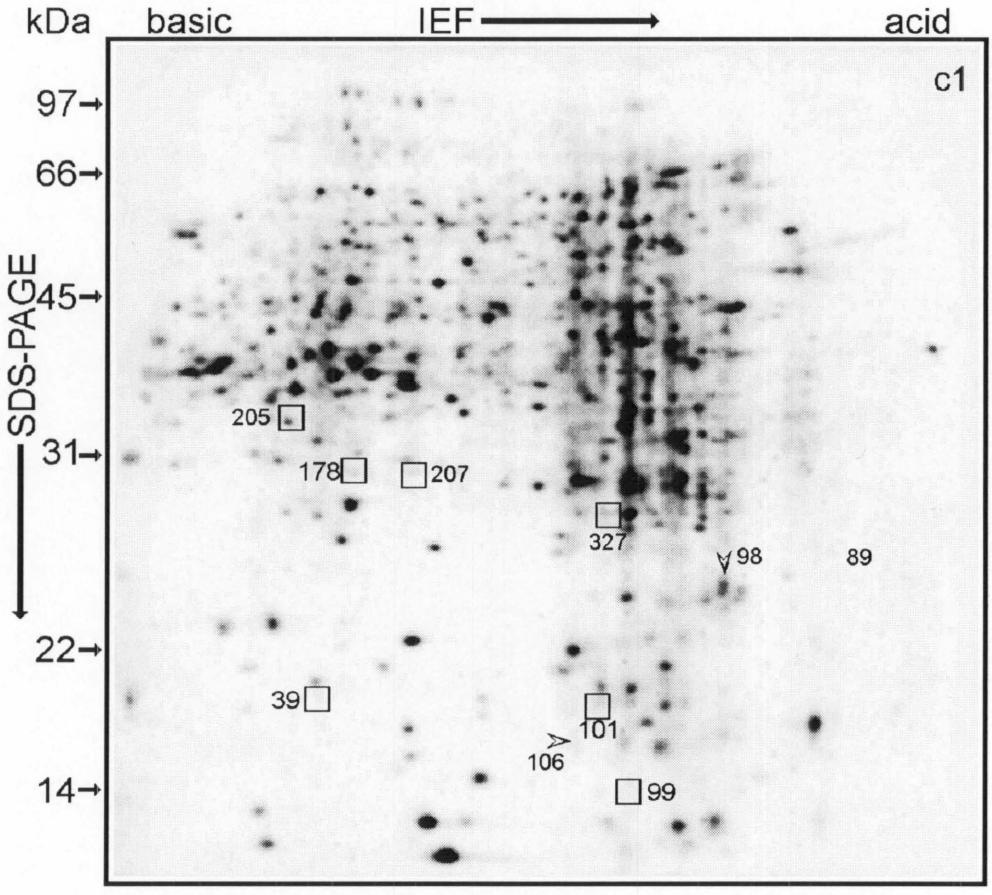

Plate 3.7 Silver-stained 2D-PAGE of proteins extracted from control of rooting region in hypocotyls of *Pinus radiata* at day 1.

Proteins associated with wounding (in □) and appeared newly in control only (≻) are indicated.

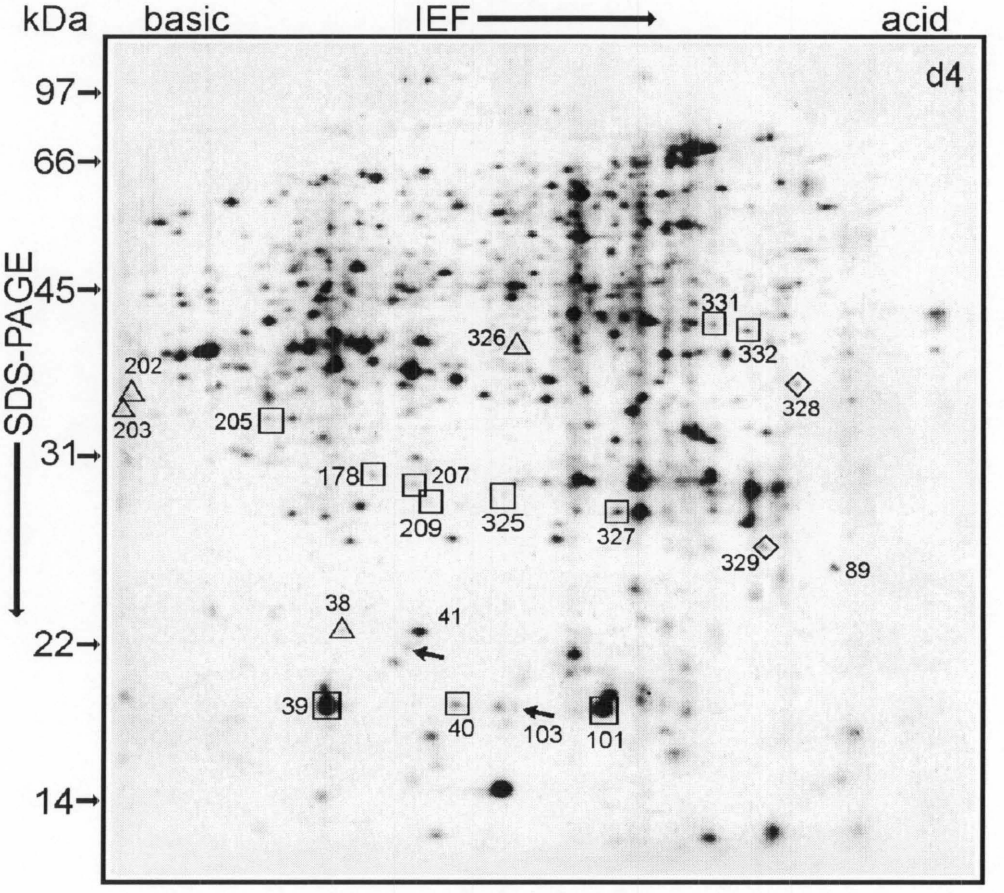

Plate 3.8 Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA at day 4.

Proteins associated with wounding (in □), induced by IBA (in △), root primordium initiation and formation (in ◇), and whole rooting process (→) are indicated.

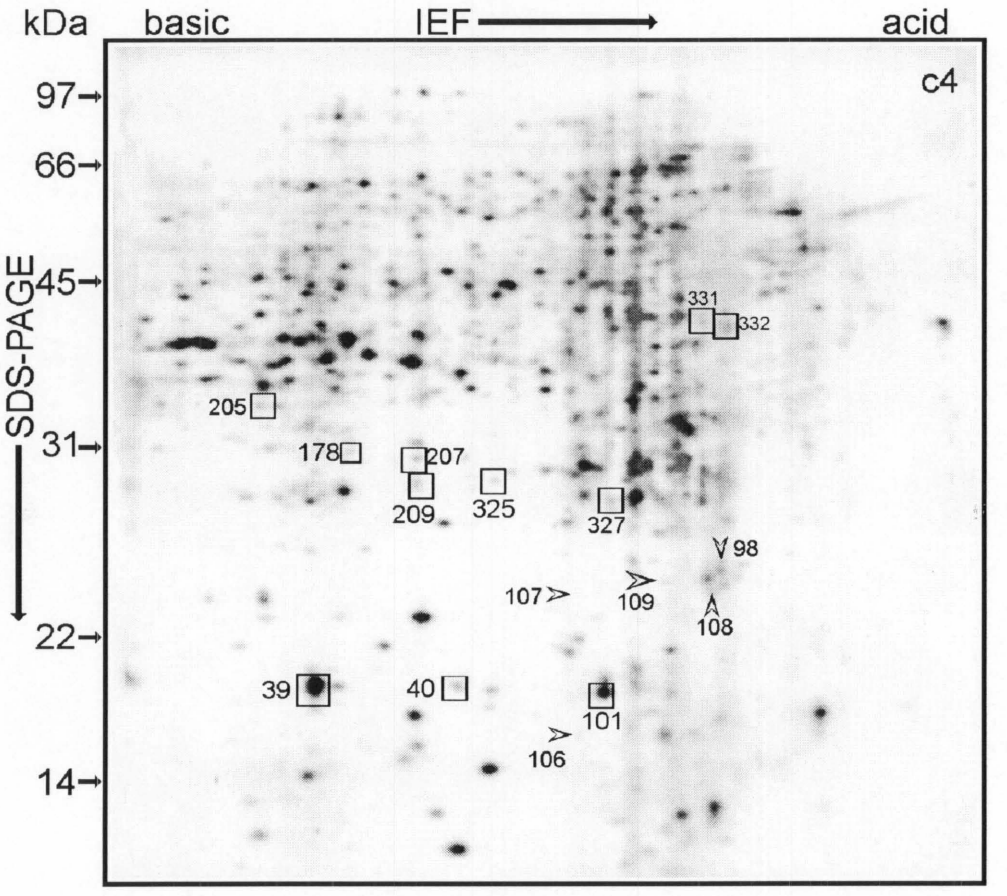

Plate 3.9 Silver-stained 2D-PAGE of proteins extracted from control of rooting region in hypocotyls of *Pinus radiata* at day 4.

Proteins associated with wounding (in □), and appeared newly in control only (➢) are indicated are indicated.

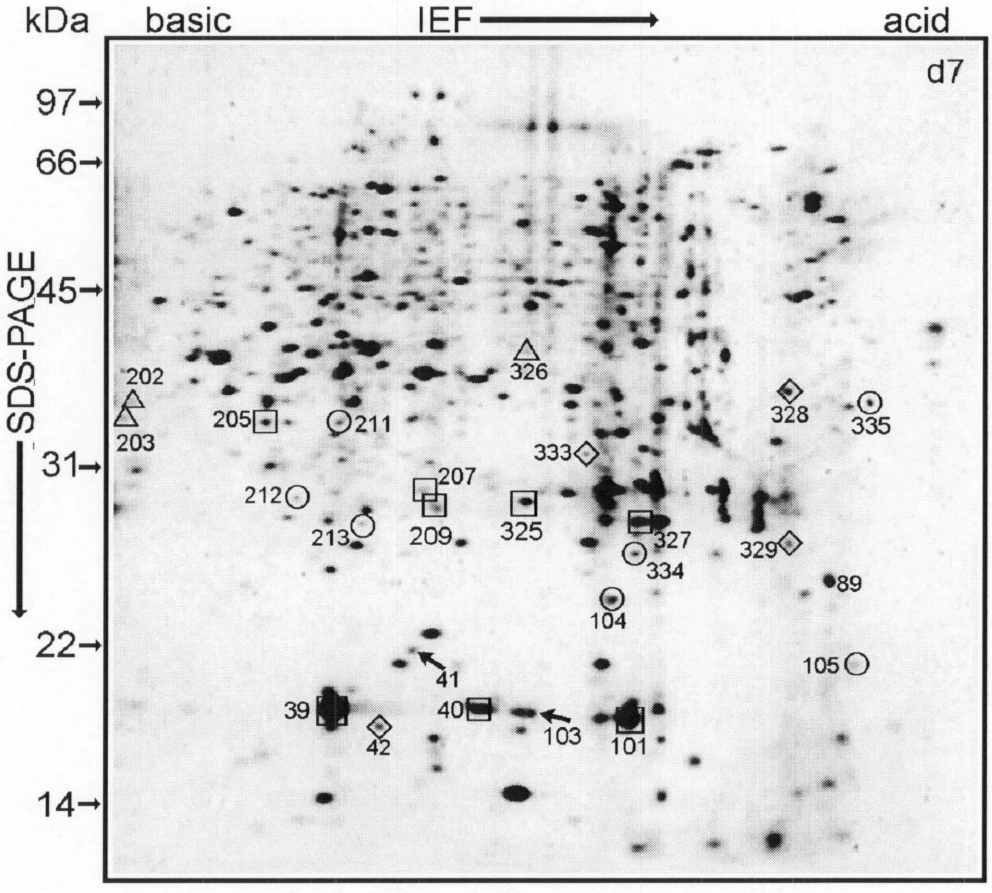

Plate 3.10 Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA at day 7.

Proteins associated with wounding (in □), induced by IBA (in △), root primordium initiation and formation (in ◇), root primordium and development (in ○), and whole rooting process (→) are indicated.

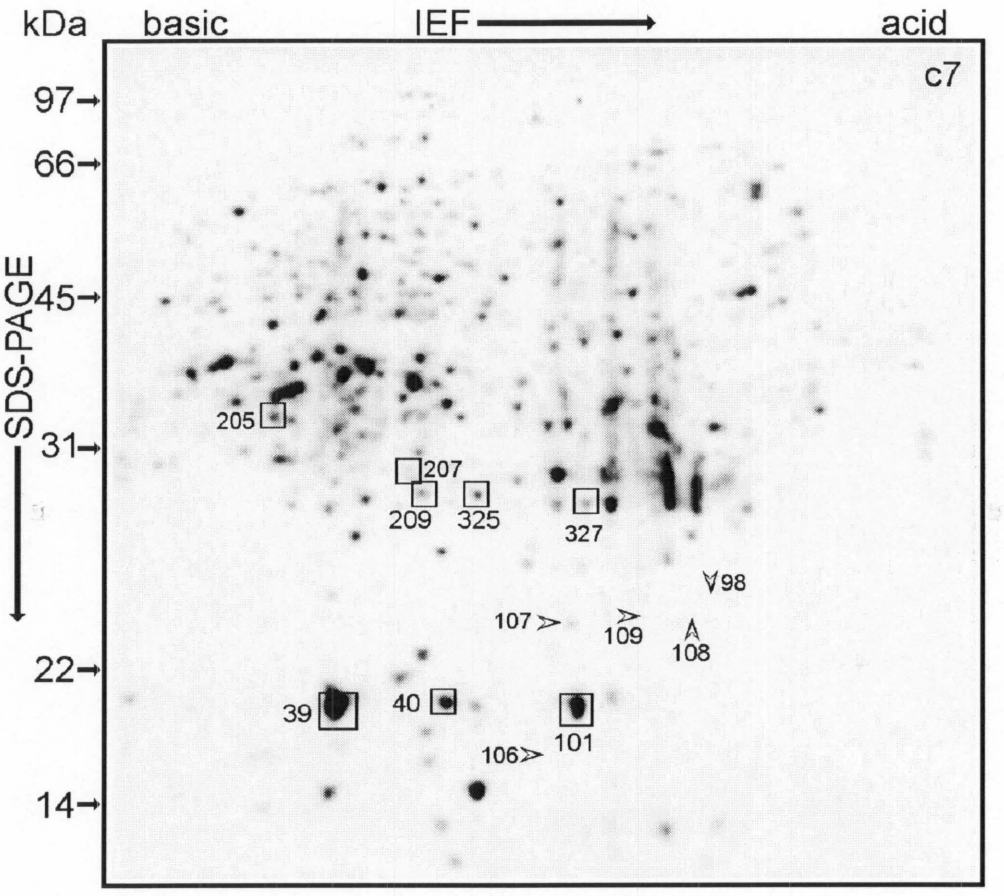

Plate 3.11 Silver-stained 2D-PAGE of proteins extracted from control of rooting region in hypocotyls of *Pinus radiata* at day 7.

Proteins associated with wounding (in □), and appeared newly in control only (≻) are indicated are indicated.

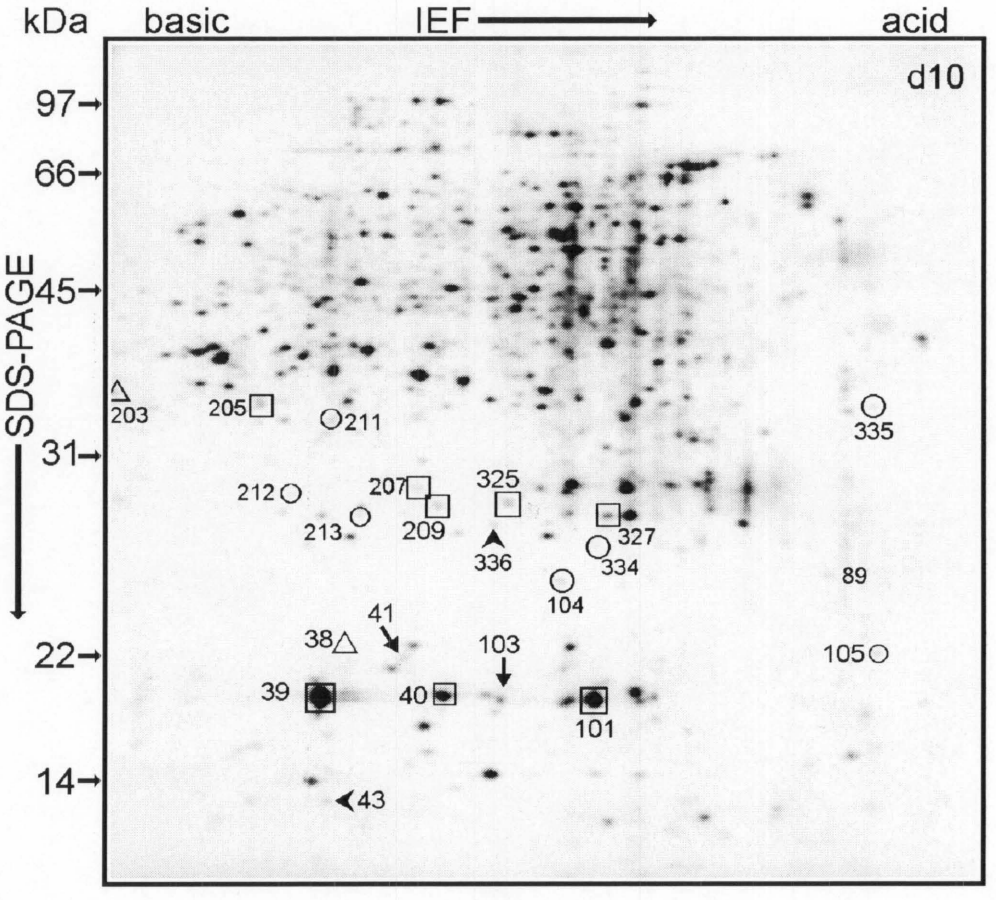

Plate 3.12 Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA at day 10.

Proteins associated with wounding (in □), induced by IBA (in △), root primordium and development (in ○), root development only (➤), and whole rooting process (→) are indicated.

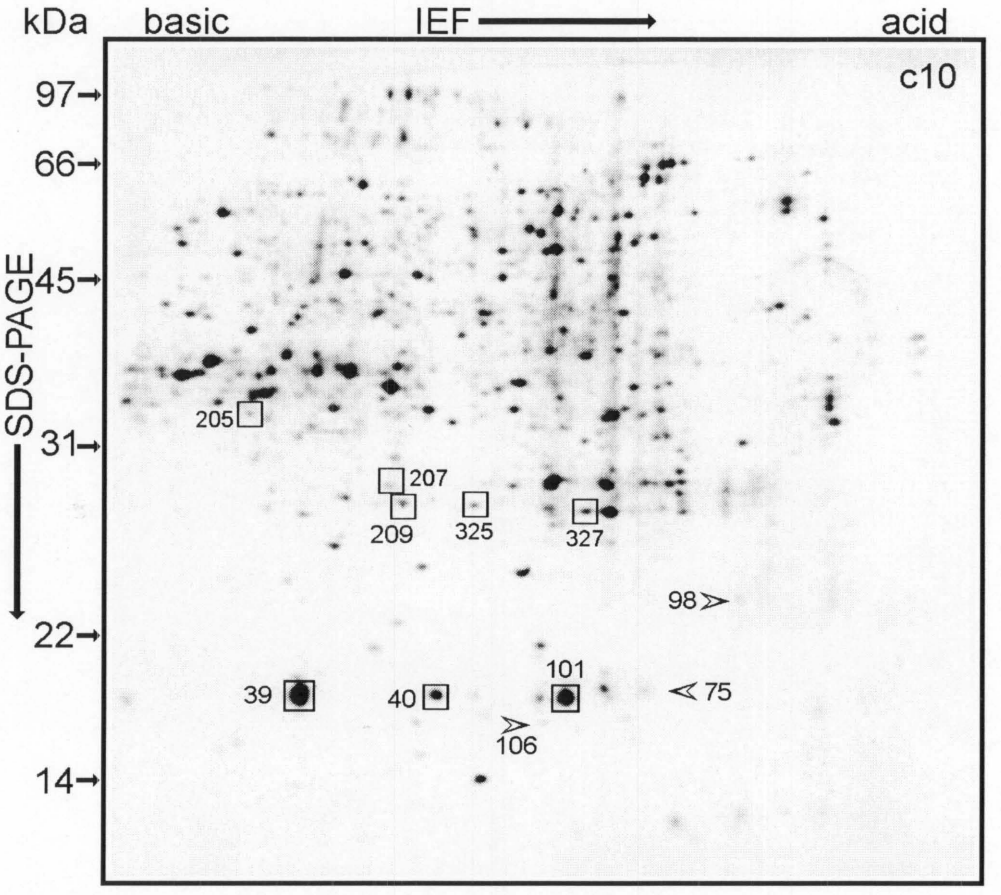

Plate 3.13 Silver-stained 2D-PAGE of proteins extracted from control of rooting region in hypocotyls of *Pinus radiata* at day 10.

Proteins associated with wounding (in □), and appeared newly in control only (≻) are indicated are indicated.

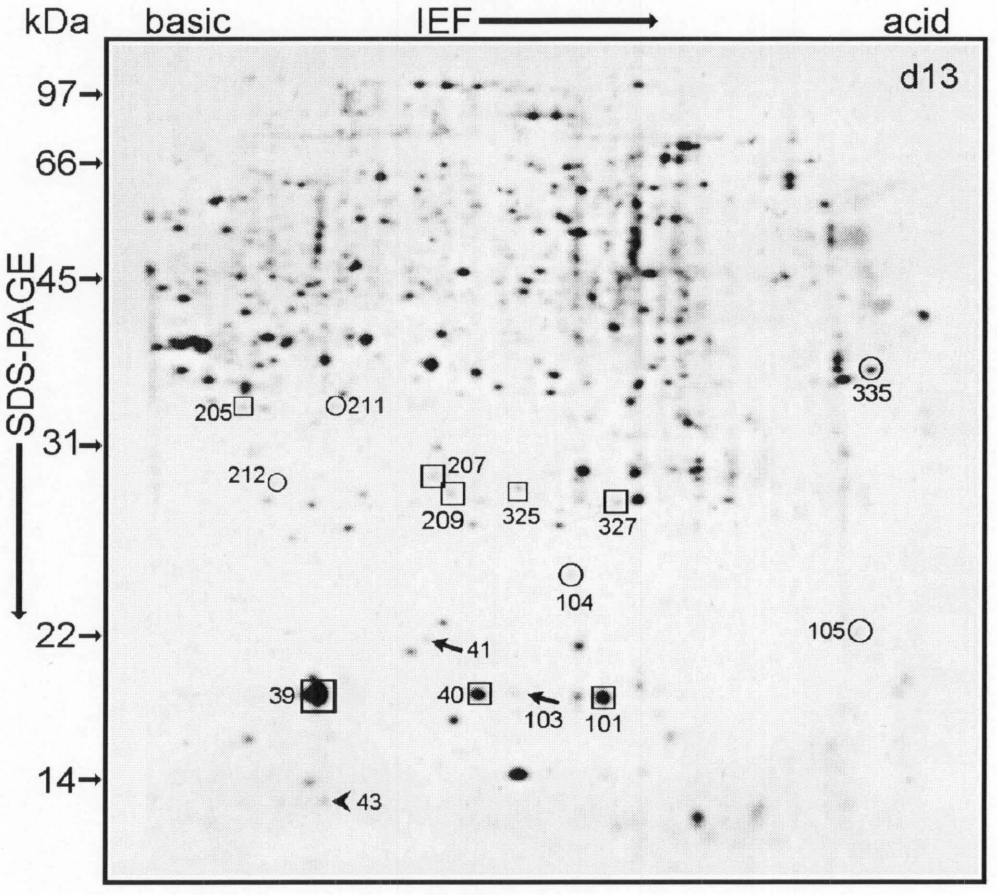

Plate 3.14 Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of *Pinus radiata* treated with IBA at day 13.

Proteins associated with wounding (in □), root primordium and development (in ○), root development only (➤), and whole rooting process (→) are indicated.

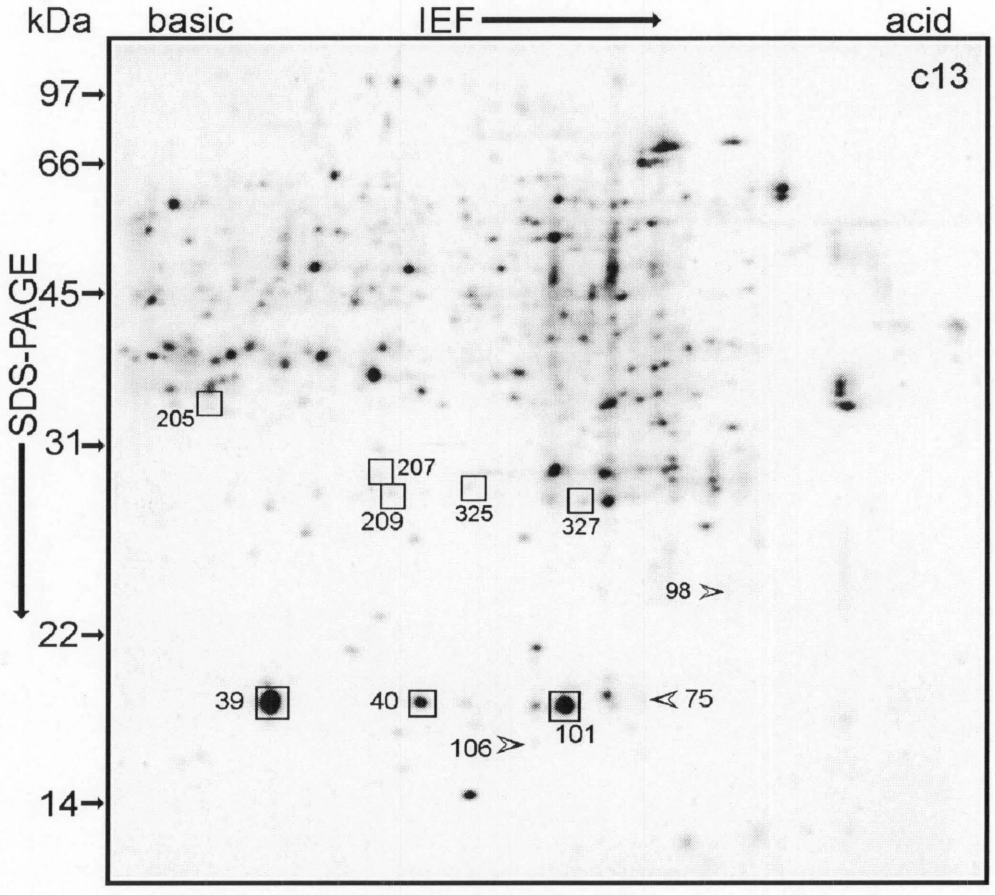

Plate 3.15 Silver-stained 2D-PAGE of proteins extracted from control of rooting region in hypocotyls of *Pinus radiata* at day 13.

Proteins associated with wounding (in □), and appeared newly in control only (≻) are indicated are indicated.

3.5 Changes of carbohydrate content

3.5.1 Changes of free sugar content

Free sugar dramatically increased during the first day in all treatments to different levels which remained relatively high throughout the experiment (Figure 3.5). This initial increase and the maintenance of higher levels of free sugar contents in the different treatments after the start of culturing is likely to be dependent on the presence of sucrose rather than the type of plant growth regulator in media (Figure 3.6). However, it appears that sugar content was higher in IBA treatment than in the others throughout the process, suggesting that somehow it might be associated with root formation. This is consistent with the findings of many researchers (see review by Haissig, 1974b).

3.5.2 Changes of starch content

3.5.2.1 Starch content in rooting region

Interestingly, starch content was very low at day 0 but increased sharply during the first day, where the increase remained almost constant in all treatments except in IBA (Figure 3.7). Under this root-inducing condition, the increase of starch content continued till day 4, then remained constant till day 7 before subsequently declined. Actually, starch content could be higher at day 4 than that at day 7. This can be deduced by the fact that amylase activity was higher at day 4 than that at day 7 (Figure 3.13). A substantial level of starch preferentially accumulated in the rooting region of the IBA treatment without kinetin and not in the non-root-inducing treatments at a critical time during the rooting process. This suggests that starch accumulation prior to root primordium organization in rooting part could have potential as a biochemical marker of this differentiation event in at least radiata pine. This postulate is consistent with the conspicuous absence of such predominance of starch accumulation in the non-root-forming region of the hypocotyls in the IBA treatment (Figure 3.8).

3.5.2.2 Starch content in the non-rooting region of IBA treated hypocotyls

Overall, starch levels in all treatments were lower in the non-root-forming than in the root-forming region of the radiata pine hypocotyl (Figure 3.8). This further proved that starch accumulation in the rooting region is a reliable marker for rooting. In the treatments with IBA+kinetin and kinetin alone, however, the starch contents have exhibited a reverse trend till day 7, i.e. the starch content of treatment with IBA+kinetin decreased to its minimum at day 4 and then increased at day 7, while the starch content of treatment with kinetin alone increased to the maximum at day 4 and decreased at day 7.

3.5.2.3 Starch content in rooting-region of hypocotyls cultured in sucrose-free medium

Starch content was also much lower in the basal region of hypocotyls cultured in sucrose-free medium (Figure 3.9), compared with the same portion of hypocotyls cultured in sucrose medium (Figure 3.7). Starch content in control (both IBA and sucrose free) increased slightly during the time course. While in IBA treatment, in which root primordia formed but most of them failed to develop into functional roots, the starch content increased to its maximum at day 7 and then decreased.

Unlike Figure 3.7, the peak of the starch content is not so pronounced as in the treatment with IBA. Therefore, statistic analysis (one way ANOVA) was carried out. Tukey's method was used to separate the means of samples following ANOVA. Initially, the significant difference was determined at $P<0.05$. In control, the slight increase of starch content was not significant until day 10 vs. day 0, while there was no significant difference among day 1 to day 13. In the treatment with IBA, however, the increase of starch content was statistically significant from day 1 onward (vs. day 0), in particular there was significant difference between day 7 and all other times (Figure 3.9). The significant difference was further tested at the level of $P<0.01$. At this level, there was not a significant difference among the starch contents of the treatment with IBA at day 4, 7 and 10, but the starch content of day 7, with IBA, was significantly different from all the others.

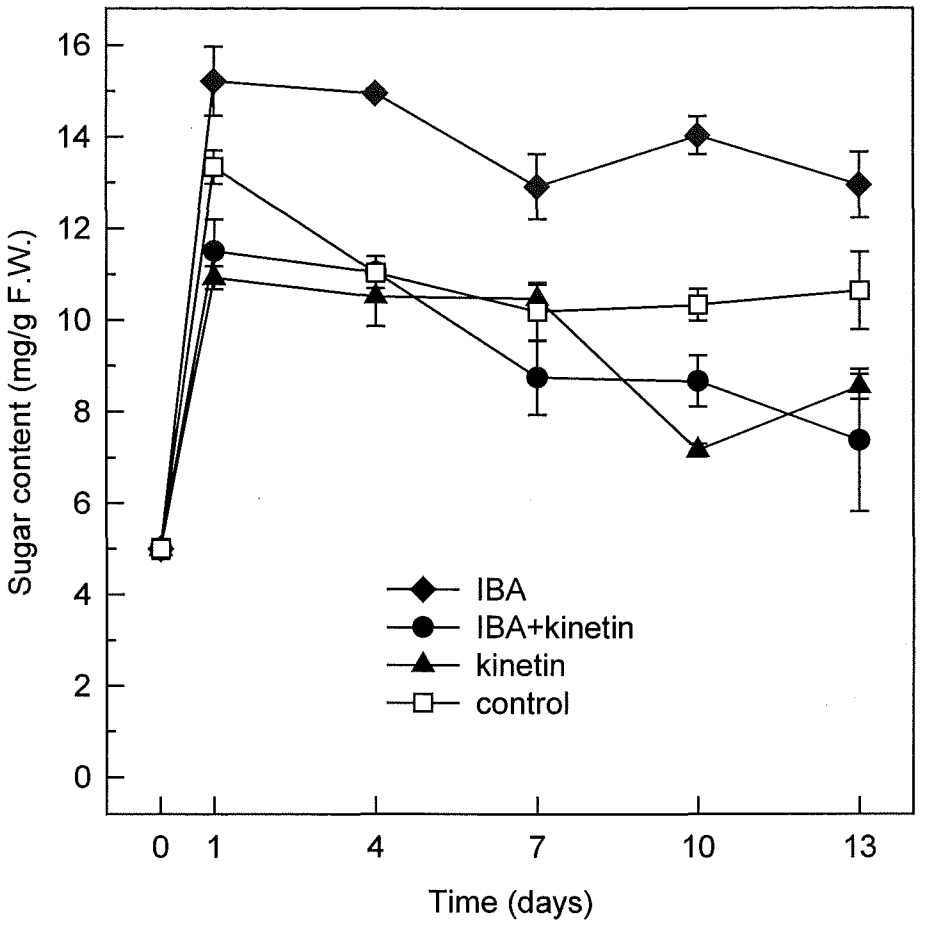

Figure 3.5 Changes of sugar content in the hypocotyl rooting region of *Pinus radiata* during rooting process.

Vertical bars represent mean ± SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

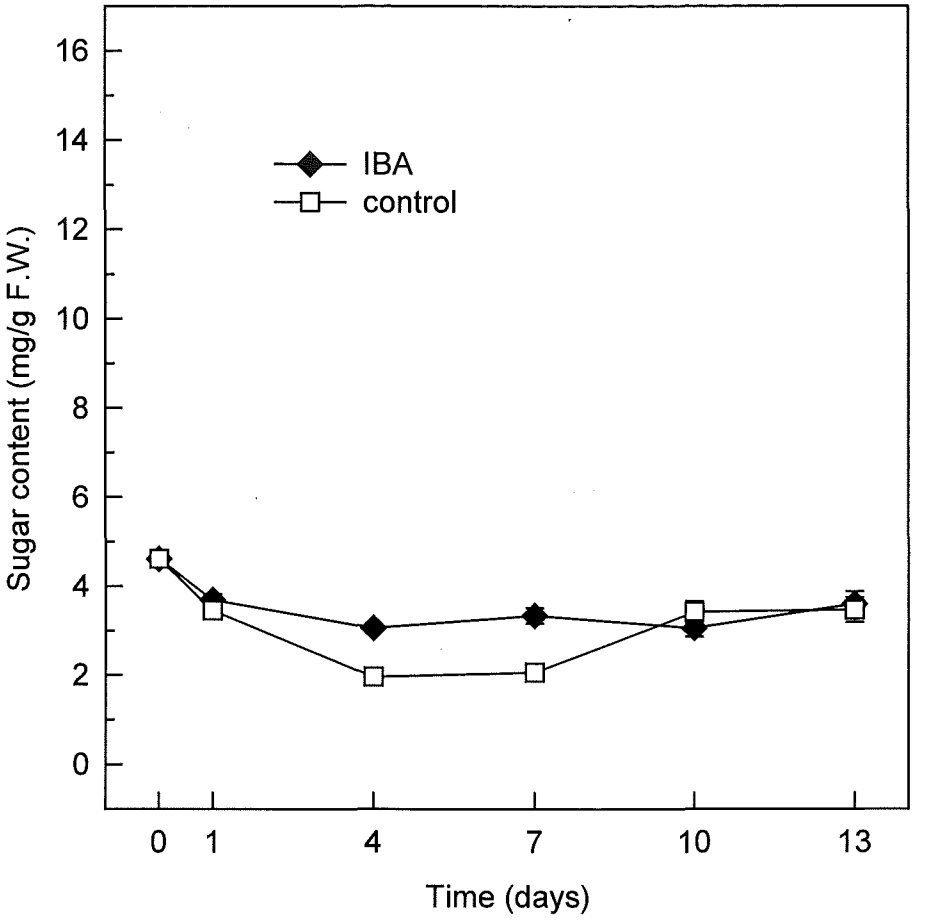

Figure 3.6 Changes of sugar content in the hypocotyl rooting region of *Pinus radiata* cultured in sucrose-free medium during rooting process.

Vertical bars represent mean ± SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

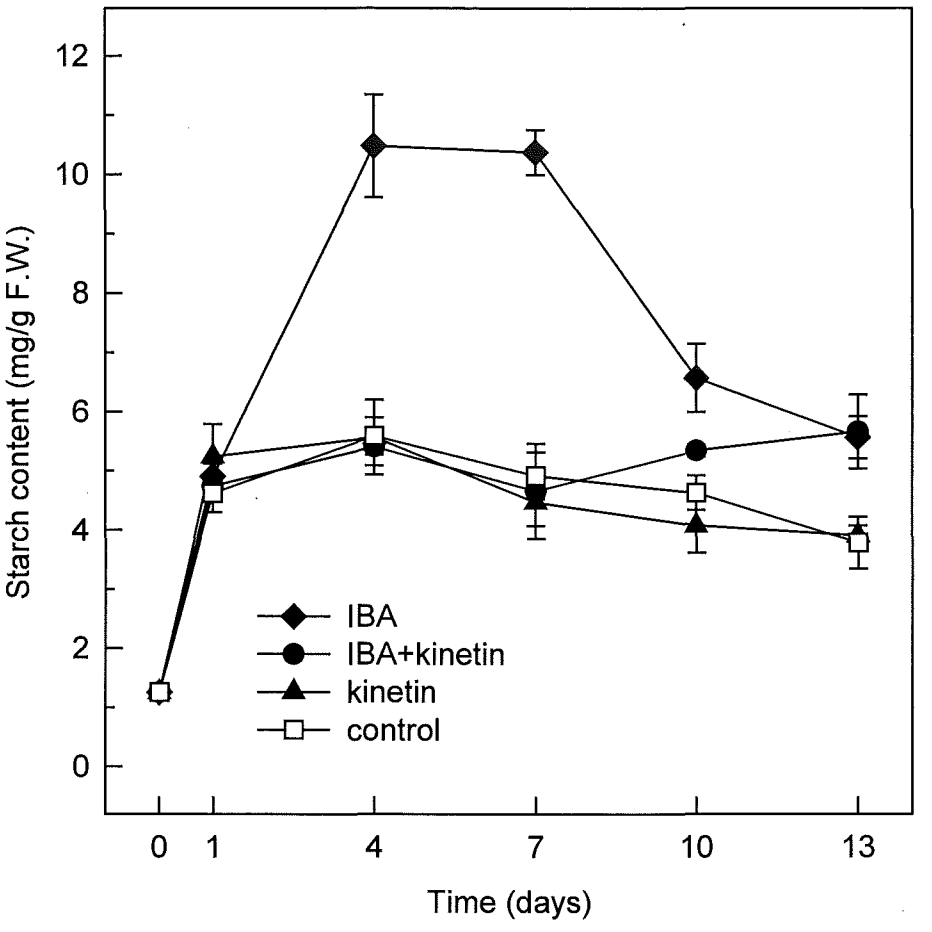

Figure 3.7 Changes of starch content in the hypocotyl rooting region of *Pinus radiata* during rooting process.

Vertical bars represent mean ± SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

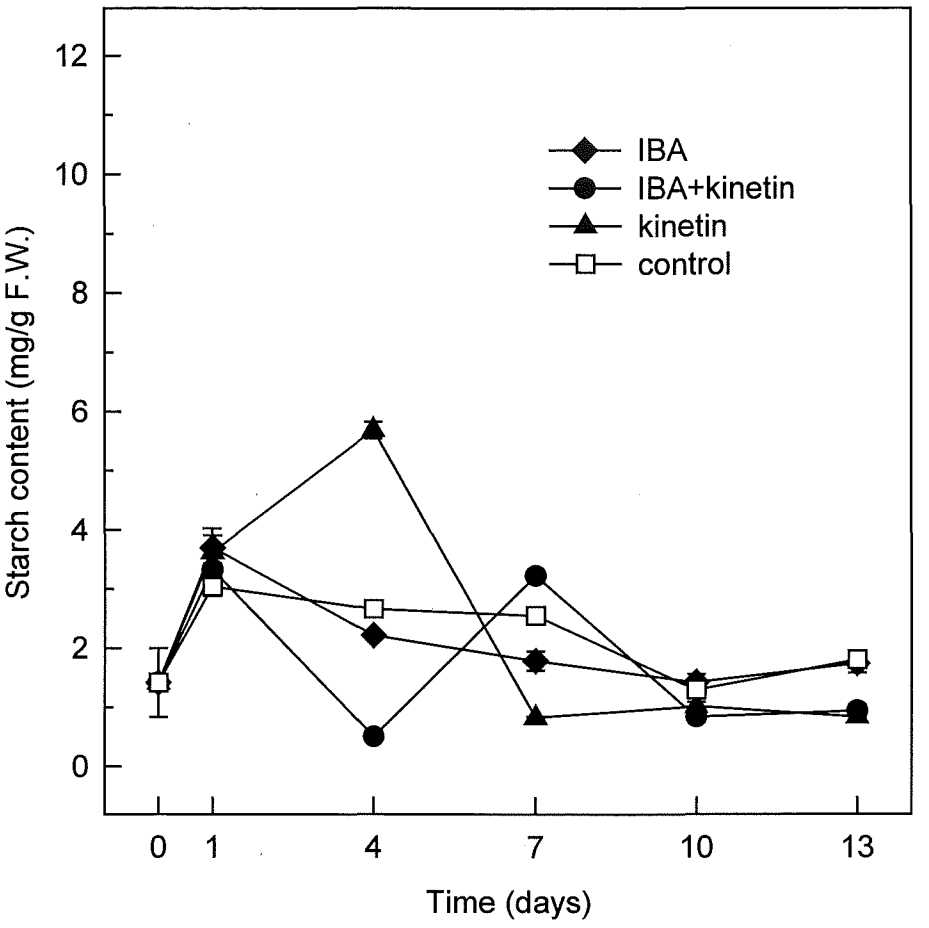

Figure 3.8 Changes of starch content in the hypocotyl non-rooting region of *Pinus radiata* during rooting process.

Vertical bars represent mean ± SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

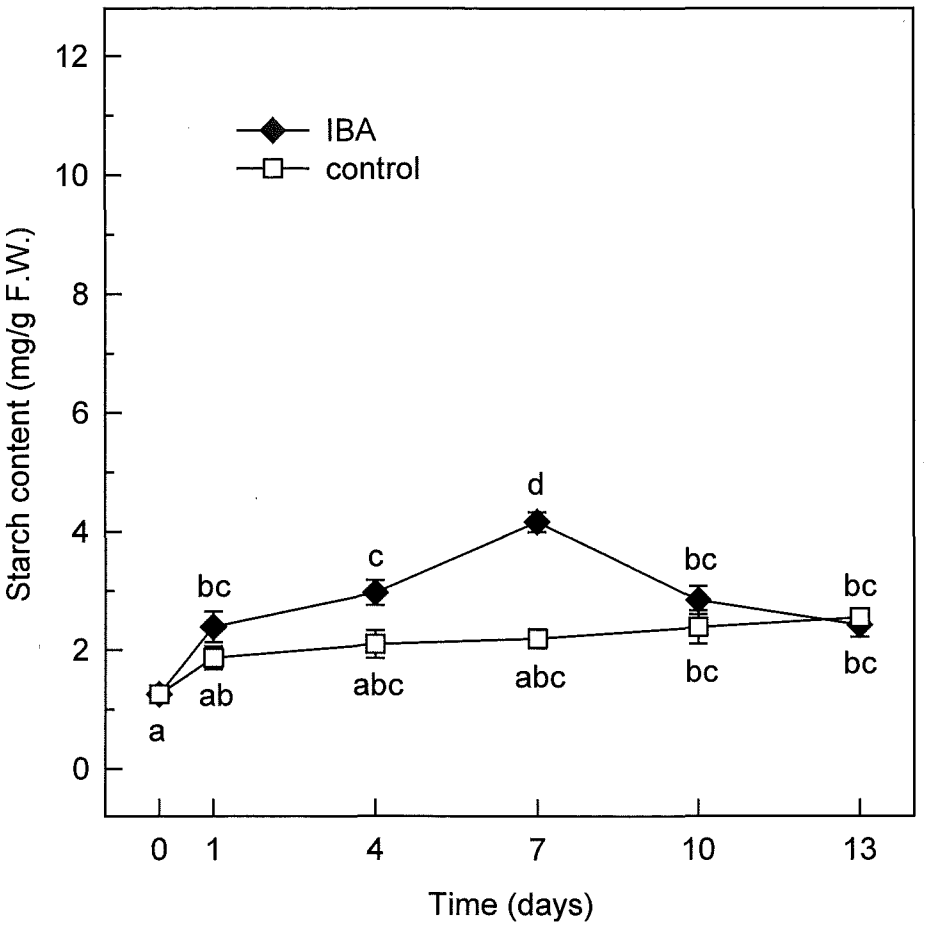

Figure 3.9 Changes of starch content in the rooting region of *Pinus radiata* hypocotyl cultured in sucrose-free medium during rooting process.

Vertical bars represent mean ± SE value for 3 determinations from 3 individual extracts. The bars with different letters are significantly different using Tukey's method ($P<0.05$). Where no bars are shown they are smaller than the legend symbols.

3.6 Changes in enzyme activities

3.6.1 Peroxidase (PO) activity

PO activity was quite high at the time when the cuttings were made and increased further during the first day, the increase being more pronounced in IBA, kinetin and control than that in IBA+kinetin. From day 4 onwards, the levels of PO activity were very similar among all 4 treatments and changed little (Figure 3.10).

3.6.2 Polyphenol oxidase (PPO) activity

PPO activity increased in all 4 treatments during the first 4 days, and continued to increase more substantially only in the treatment with IBA (Figure 3.11). This increase led to a peak of PPO activity in this treatment, higher than those of the 3 other treatments. The enzyme activity in the IBA treatment then decreased somewhat until the end of the rooting process.

3.6.3 IAA-oxidase (IAA-O) activity

IAA-O activity was reasonably low at the time when the cuttings were made (day 0) and increased gradually in all 4 treatments throughout the experimental period, with the exception of a decrease in kinetin at day 13 (Figure 3.12). IAA-O activity in the treatment with IBA increased from 0.18 to 1.00 Unit during the rooting process, with three fractions of linear increases (day 0-4, day 4-7 and day 7-13). The activity in control was very close to that in the treatment with IBA before day 7, but the difference became obvious thereafter, especially during day 10-13. IAA-O activities in the treatments with IBA+kinetin and kinetin alone were very similar, with an exception of a decrease in the treatment with kinetin alone at day 13. In this study, modified Salkowski reagent (Gordon & Weber, 1951) was used to give more reliable results as a more stable colour was produced with increased specificity and sensitivity. Standard errors were always very small.

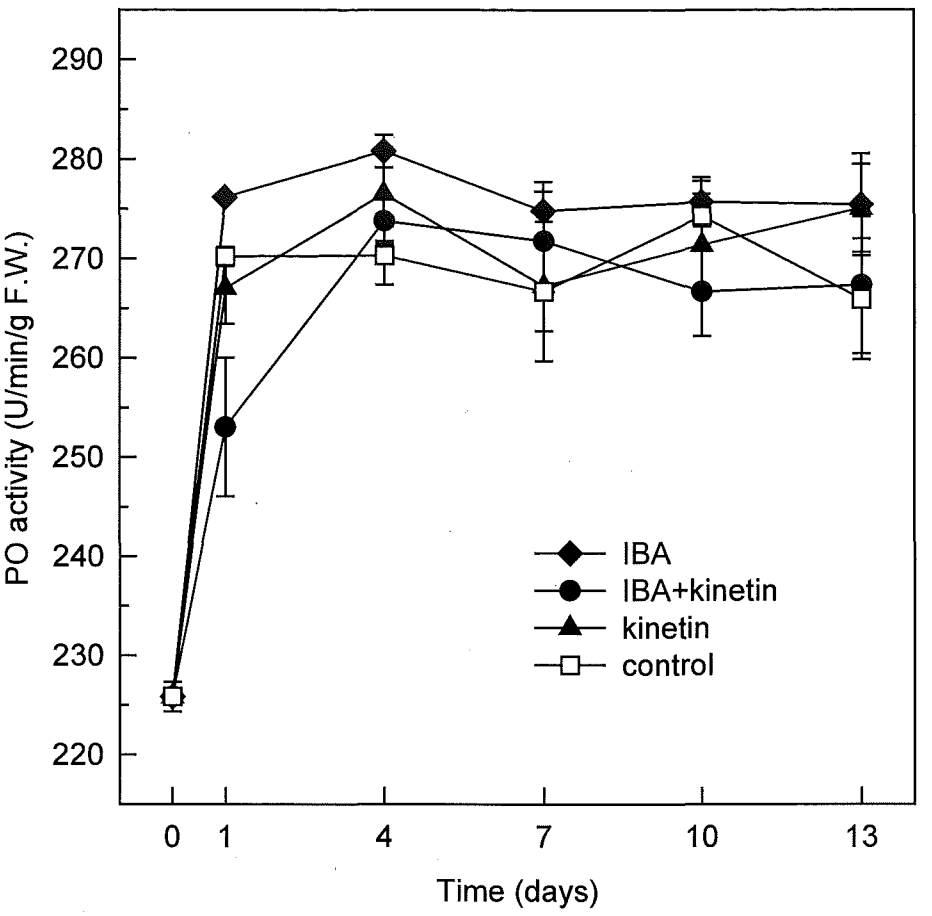

Figure 3.10 Changes of PO activity in the rooting-region of hypocotyls in *Pinus radiata.*

Vertical bars represent mean + SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

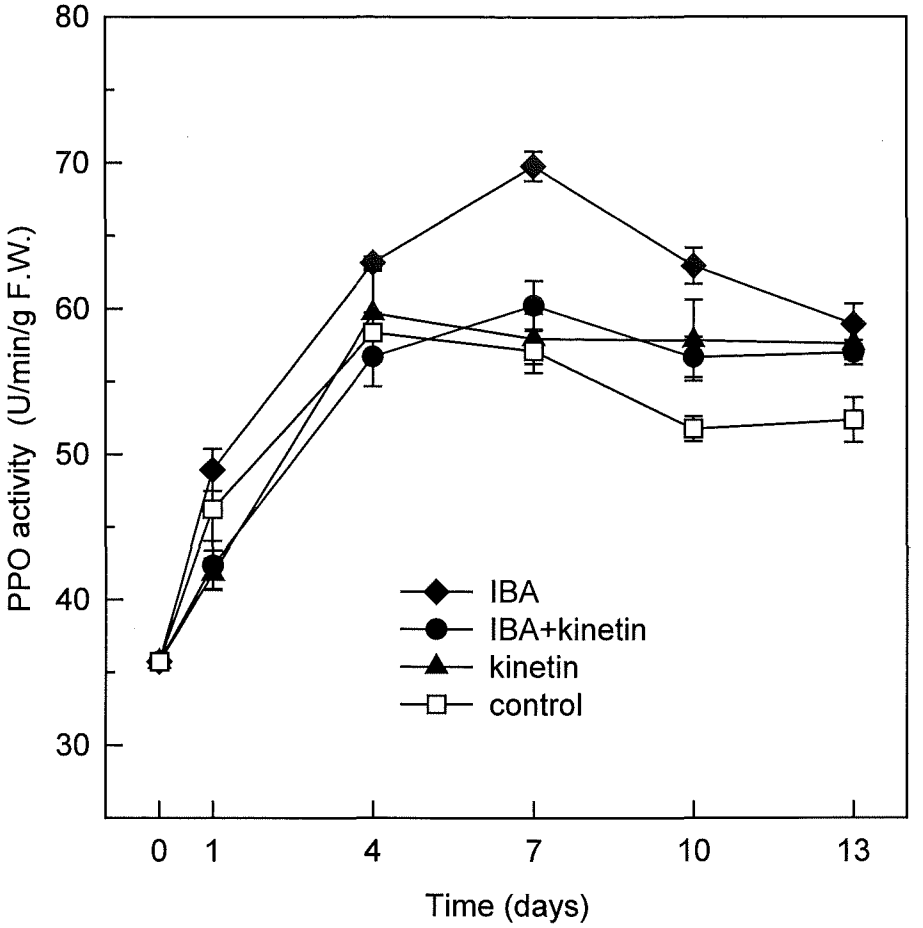

Figure 3.11 Changes of PPO activity in the rooting-region of hypocotyls in *Pinus radiata.*

Vertical bars represent mean + SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

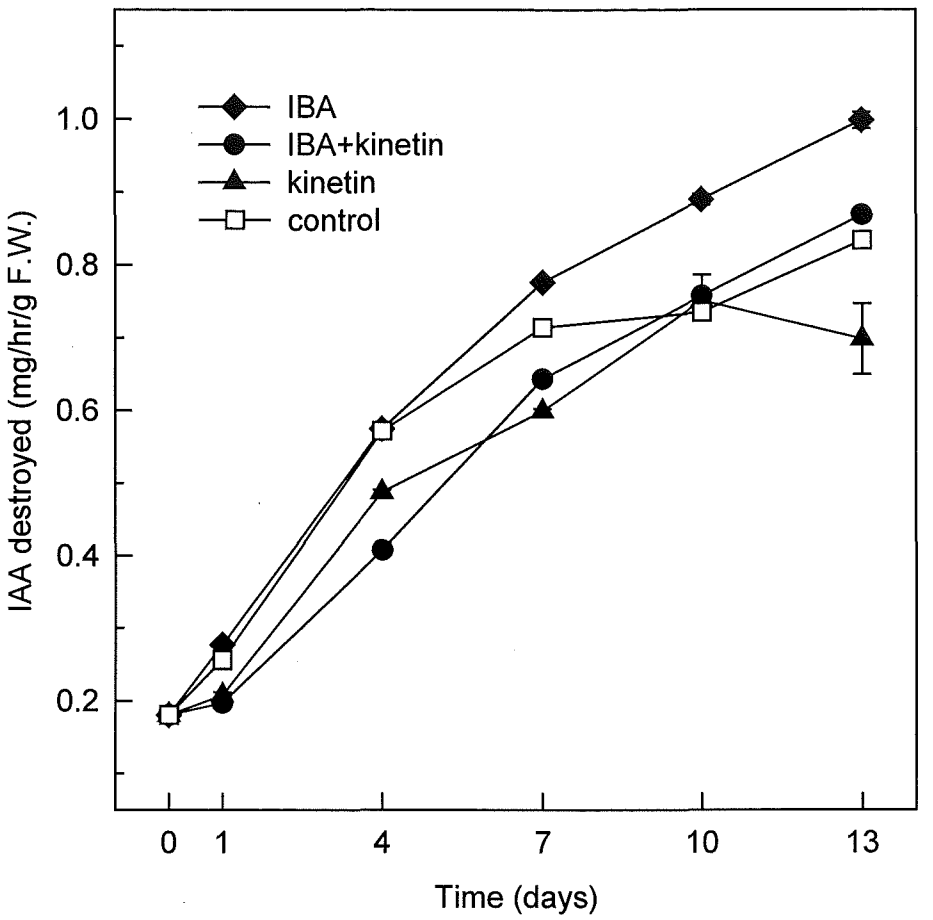

Figure 3.12 Changes of IAA-O activity in the rooting-region of hypocotyls in *Pinus radiata*.

Vertical bars represent mean + SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

3.6.4 Amylase (Amy) activity

Amylase activity gradually decreased in control, this pattern being completely different from the others (Figure 3.13). In both IBA and IBA+kinetin, the activity slightly decreased during the first day, subsequently increased during day 1-4 and decreased during day 4-7, then remained constant till day 10 and increased again till day 13. The pattern of amylase activity in the kinetin treatment is also same as that of IBA and IBA+kinetin before day 7 before it increased sharply during day 7-10.

3.6.5 Succinic dehydrogenase (SDH) activity

SDH activity increased markedly in all 4 treatments during the first day, the increase being smaller in the treatment with IBA while the other three treatments had similar levels (Figure 3.14). Then, the overall trend was that the levels of SDH activities dropped throughout the experimental period, except that the decrease in the control was delayed till after day 4.

3.7 Histochemistry

3.7.1 Starch localization

At the time when the cuttings were made, most cells were devoid of starch, except for some endodermis cells (Plate 3.16-A). Starch grains were completely absent from cortex cells. Initially, starch accumulation (dark spots) occurred in the cells of endodermis and pith in the hypocotyl cuttings treated with IBA at day 1 (Plate 3.16-B). At day 4, starch deposition was more pronounced in the hypocotyl cuttings treated with IBA (Plate 3.16-C) than the non-rooting control (Plate 3.16-D), particularly in the pith and the area around differentiating resin ducts or so-called inner cortex (Smith & Thorpe, 1975a). In contrast, much fewer starch grains were detected in cortex (Plate 3.16-C and D).

Disappearance of the accumulated starch grains seemed to be associated with the initiation of meristematic tissue and primordia within the IBA-treated hypocotyls. At

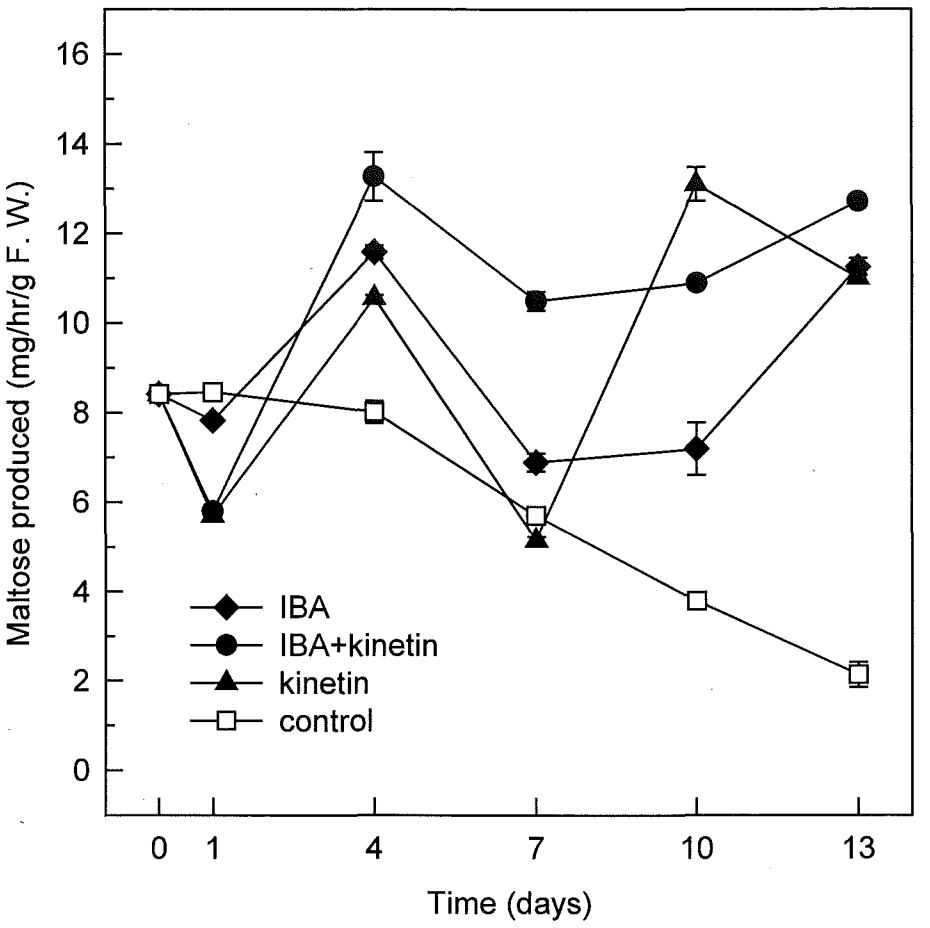

Figure 3.13 Changes of amylase activity in the rooting-region of hypocotyls in *Pinus radiata*.

Vertical bars represent mean + SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

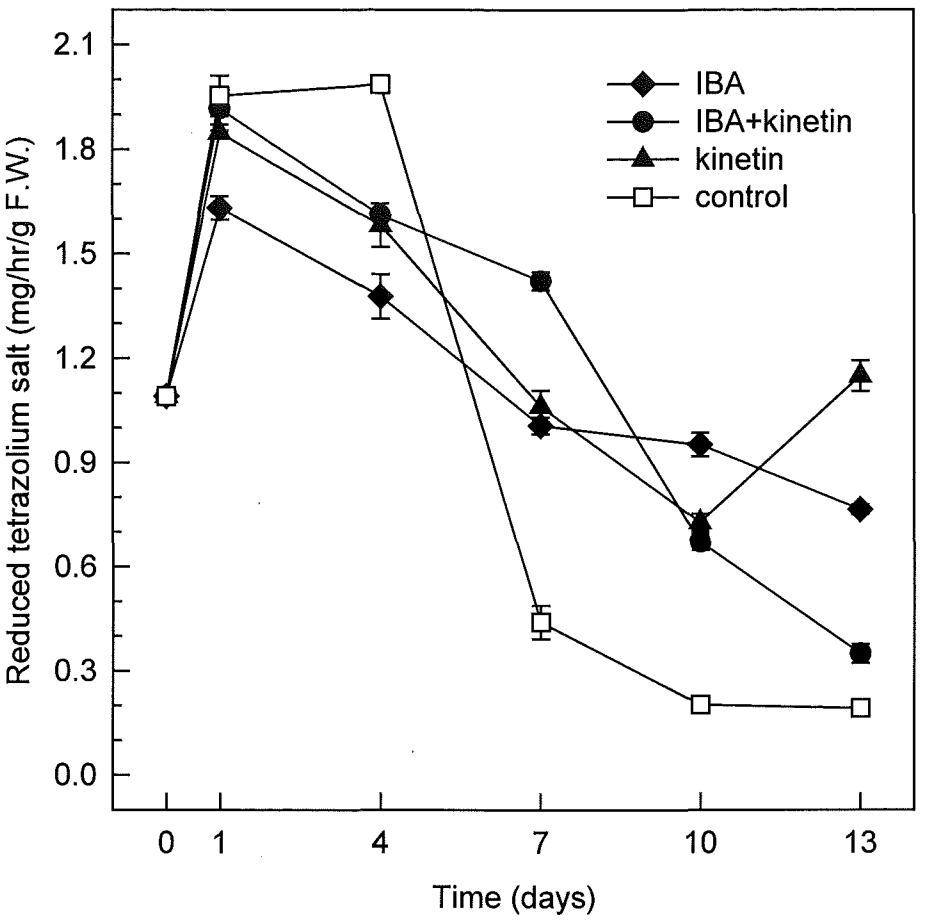

Figure 3.14 Changes of SDH in the rooting-region of hypocotyls in *Pinus radiata.*

Vertical bars represent mean + SE value for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the legend symbols.

day 7, starch grains were still preferentially associated with the newly formed putative root primordium tissue within the hypocotyl cuttings treated with IBA, but were at reduced levels in the pith and at the periphery of the meristematic tissues (Plate 3.16-E). In the hypocotyl cuttings of non-rooting control, the starch content was apparently similar to that at day 4, except a little decrease in the cells of endodermis (Plate 3.16-F). At day 10 when the root primordia were well formed, the starch level in the pith and the periphery of root primordia appeared to drop further although some starch grains were still associated with the primordia (Plate 3.16-G). These starch grains are expected to be used for the further development of the adventitious roots. Very few starch grains were observed in the section from the hypocotyl cuttings of non-rooting control at this stage (Plate 3.16-H).

3.7.2 PO localization

Initially, benzidine, the most common substrate for PO detection at a histological level (van Fleet, 1959), was used and the enzyme activity was indicated by a dark blue coloration (benzidine blue). However, this reaction product was unstable as reported by other authors (van Fleet, 1959; De Jong, 1967; Molnar & LaCroix, 1972b). Benzidine, in the presence of H_2O_2 and PO, forms benzidine blue, which is broken down to benzidine brown (diimine). van Duijin (1955) reported that the reaction could be stopped in the benzidine blue stage by the use of 5% ammonium chloride in the benzidine-H_2O_2 mixture. However, it was found to be unsatisfactory in this study which is in agreement with Molnar & LaCroix (1972b). Therefore, in most cases PO activity was indicated by a brown coloration while the dark blue coloration was occasionally observed in some tissues.

PO activity was localized at the epidermis at the time when the cuttings were made (Plate 3.17-A), compared with the control sections that were stained in substrate blank mixture (Plate 3.17-B). At day 4 increased enzyme activity was observed in all tissues, including epidermis, pericycle and pith, with somewhat lower activity in the cortex (Plate 3.17-C). In particular, meristematic loci, which were localized at the margin of the differentiating resin ducts, showed relatively strong enzyme activity.

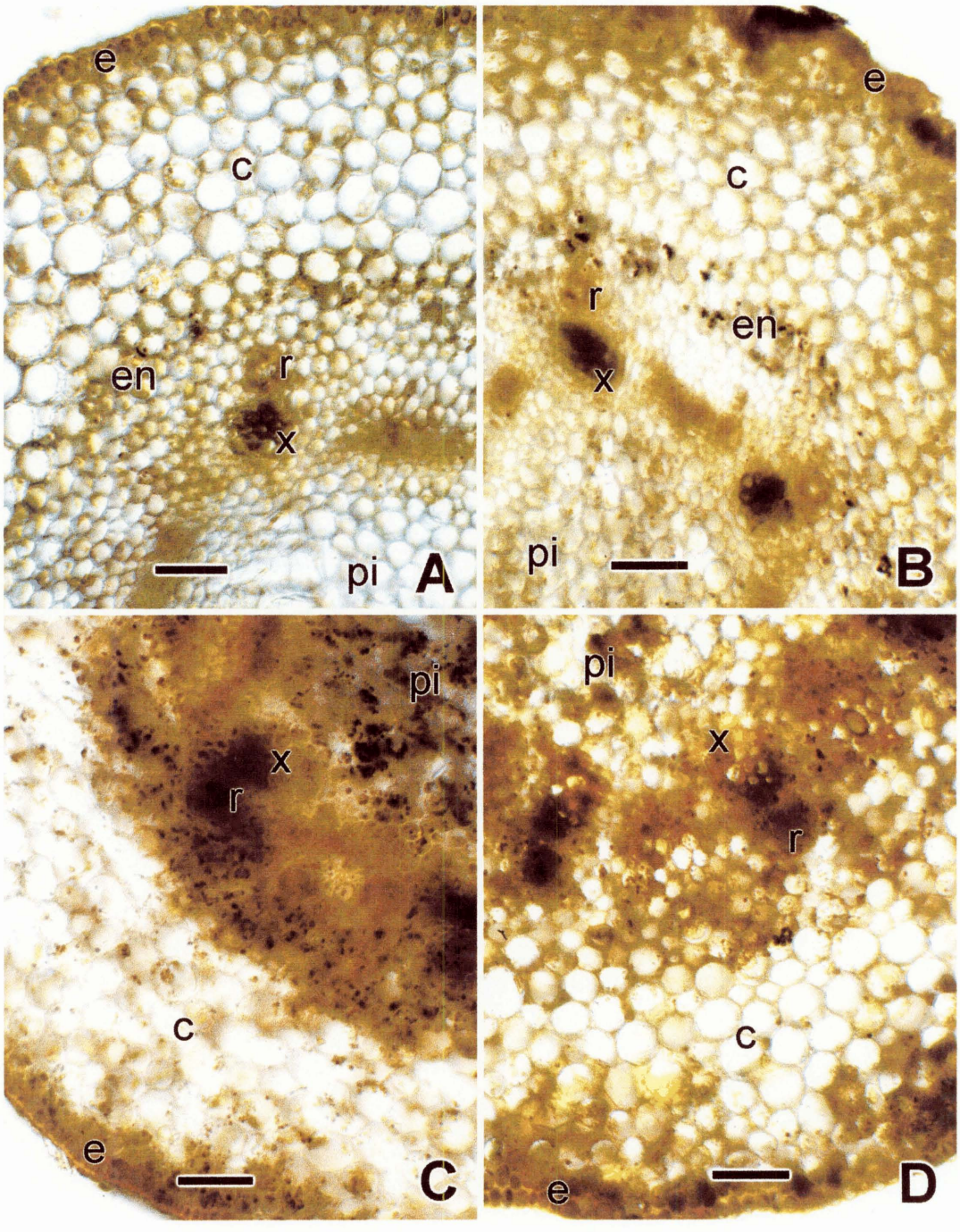

Plate 3.16 Histochemical localization of starch grains in the basal region of the hypocotyl cuttings of *Pinus radiata*.

c = Cortex, r = Differentiating resin duct, x = Protoxylem, e = Epidermis, en = endodermis. All bars = 100 μm. Transverse section at day 0 (A), at day 1 (B) and at day 4 (C) after IBA treatment, non-rooting control at day 4 (D). Starch grains are indicated as dark spots.

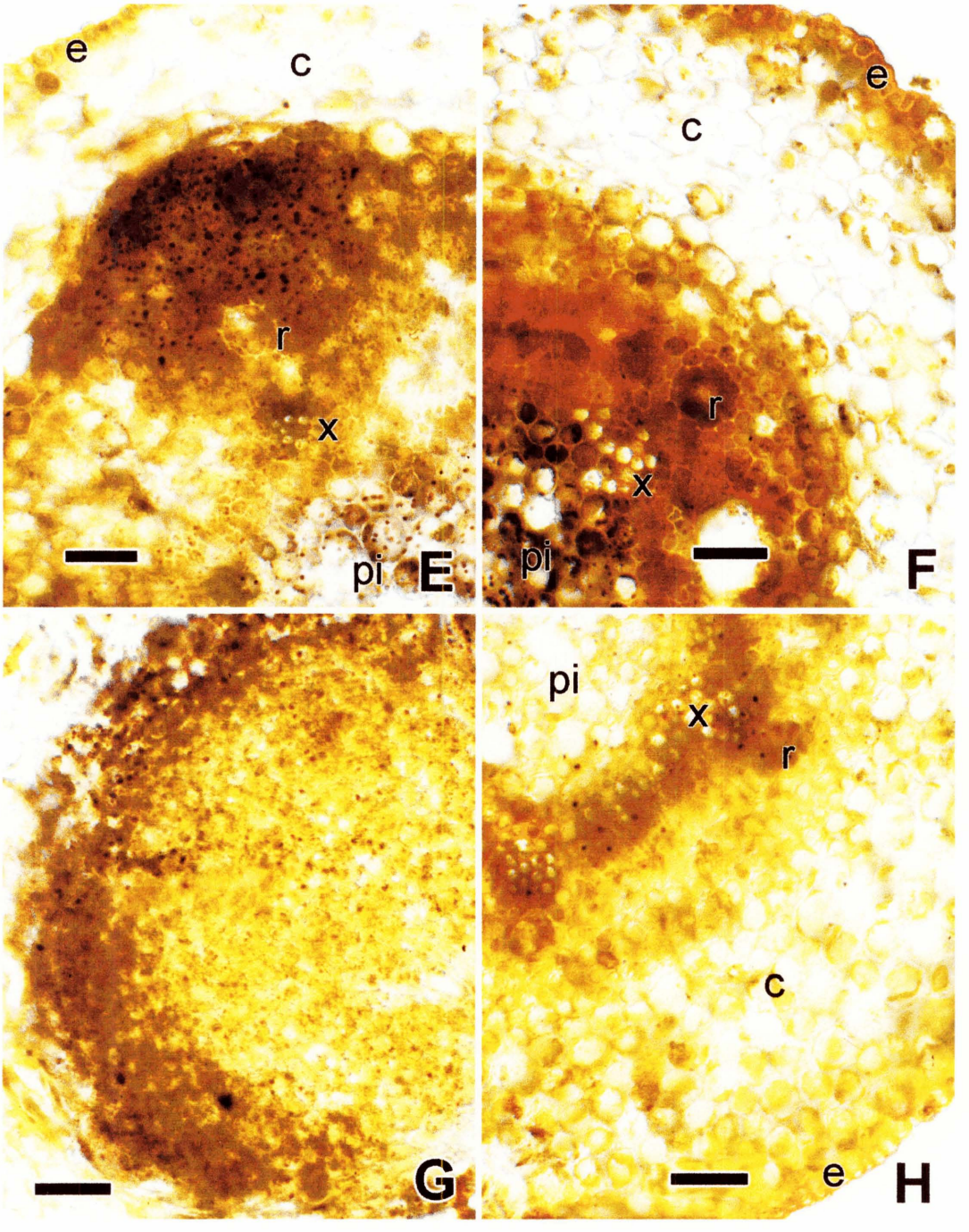

Plate 3.16 (continued) Histochemical localization of starch grains in the basal region of the hypocotyl cuttings of *Pinus radiata*.

c = Cortex, r = Differentiating resin duct, x = Protoxylem, e = Epidermis. All bars = 100 μm. Transverse section at day 7 (E) after IBA treatment and non-rooting control at day 7 (F), at day 10 (G) after IBA treatment and non-rooting control at day 10 (H). Starch grains are indicated as dark spots.

With the initiation of root primordia, the enzyme activity increased somewhat. The periphery of the meristematic tissue or primordia had the highest activity (Plate 3.17-D and E). The increased PO activity in rooting control (auxin-free), compared with day 0, was observed at epidermis, pith and interfascicular cambium (Plate 3.17-F). According to van Fleet (1959), appearance of coloration (dark blue and/or brown) was dependent on different kinds of tissues and cells. In general, it seemed that PO activity increased with the developmental sequence of root primordium formation. This, however, is not consistent with the data obtained by the colorimetric assays of tissue extract, in which the enzyme activity kept relatively constant during day 1 to 13 (Figure 3.10).

3.7.3 PPO localization

The enzyme activity was indicated by a darkening reaction, which is typical of melanin formation as a result of DOPA oxidation by PPO or tyrosinase (Smith & Thorpe, 1975a). PPO was relatively low and mainly localized at epidermis at the time when the cuttings were made (Plate 3.18-A). It is noted that black coloration in proxylem and differentiating resin ducts was not completely due to PPO as the sections of enzyme-controls also had the similar characteristic, but the colour was lighter in control. The enzyme activity increased in pericycle and pith after the cuttings were taken. It was detected at the expected sites of root initiation at day 6, a group of cells showing black coloration (Plate 3.18-B). This group of cells, meristematic tissues, became larger in size and darker in colour at day 7 (Plate 3.18-C). At the time when primordia were formed (day 10), PPO activity declined in the centres of the primordia, and high enzyme activity was located at the periphery of the primordia and interfascicular cambium (Plate 3.18-E). In the rooting-control treatment (auxin-free), the high PPO activity was detected at interfascicular cambium (Plate 3.18-D and F).

3.7.4 SDH localization

SDH reduces neotetrazolium chloride to a bright purple, water-insoluble formazan (Molnar & LaCroix, 1972b). This enzyme appeared to have very low activity in

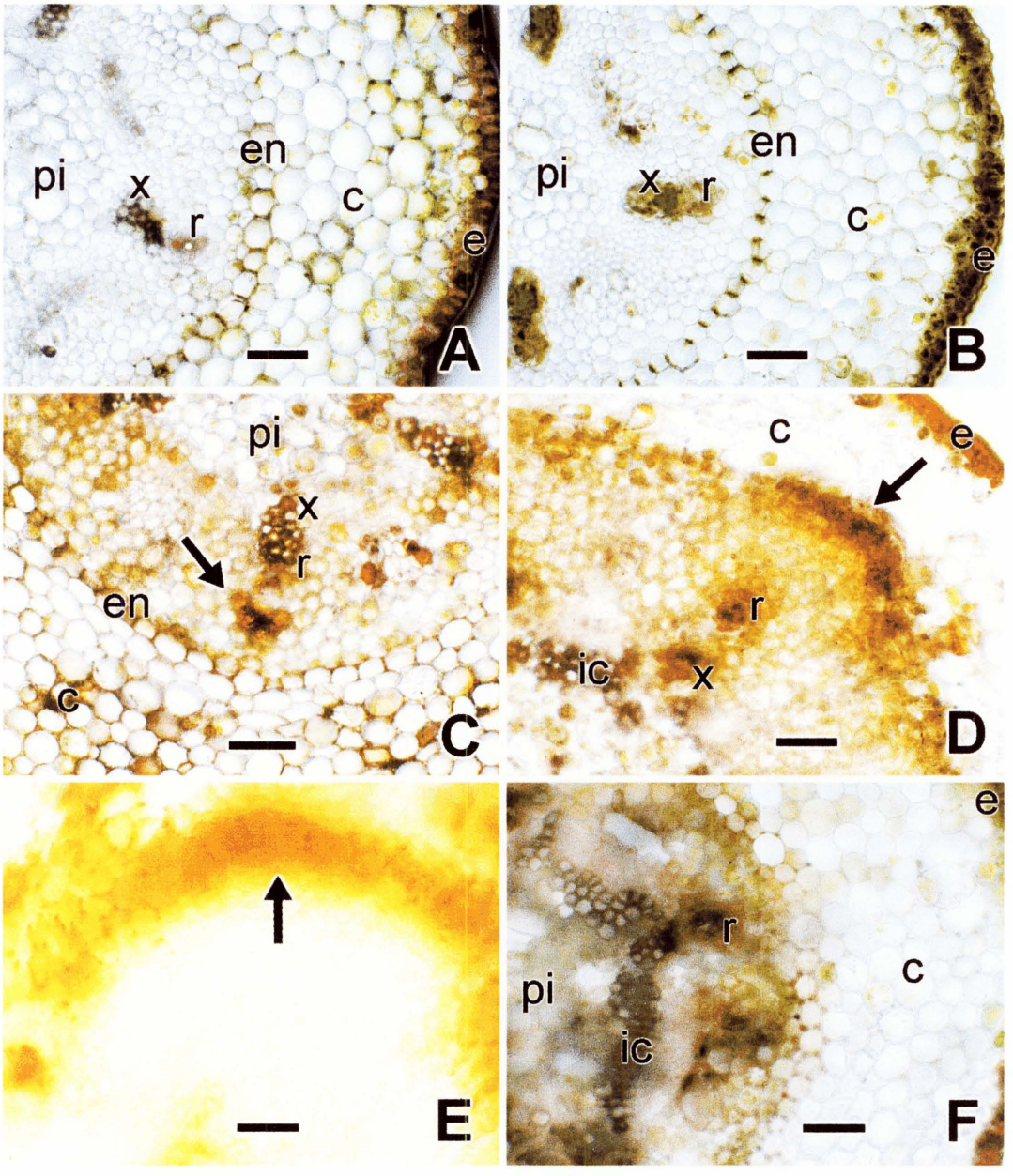

Plate 3.17 Histochemical localization of PO activity in the basal region of the hypocotyl cuttings of *Pinus radiata*.

Brown and dark blue colour indicate the enzyme activity. c = Cortex, r = Differentiating resin duct, x = Protoxylem, e = Epidermis, en = endodermis, ic = interfascicular cambium. All bars = 100 μm. (A) Transverse section at day 0. (B) Transverse section of enzyme control at day 0. (C)-(E) show sections from the IBA-rooting-inducing treatment. (C) Transverse section at day 4. Note strong PO activity at the sites of meristematic loci (arrow). (D) Transverse section at day 7. Strong PO activity is located at the periphery of meristematic tissue (arrow). (E) Transverse section at day 10. Strong PO activity is located at the periphery of the primordium (arrow). (F) Transverse section of rooting-control at day 10. Strong PO activity is located at epidermis, interfascicular cambium, and pith. Benzidine blue other than benzidine brown is indicated at interfascicular cambium, and pith.

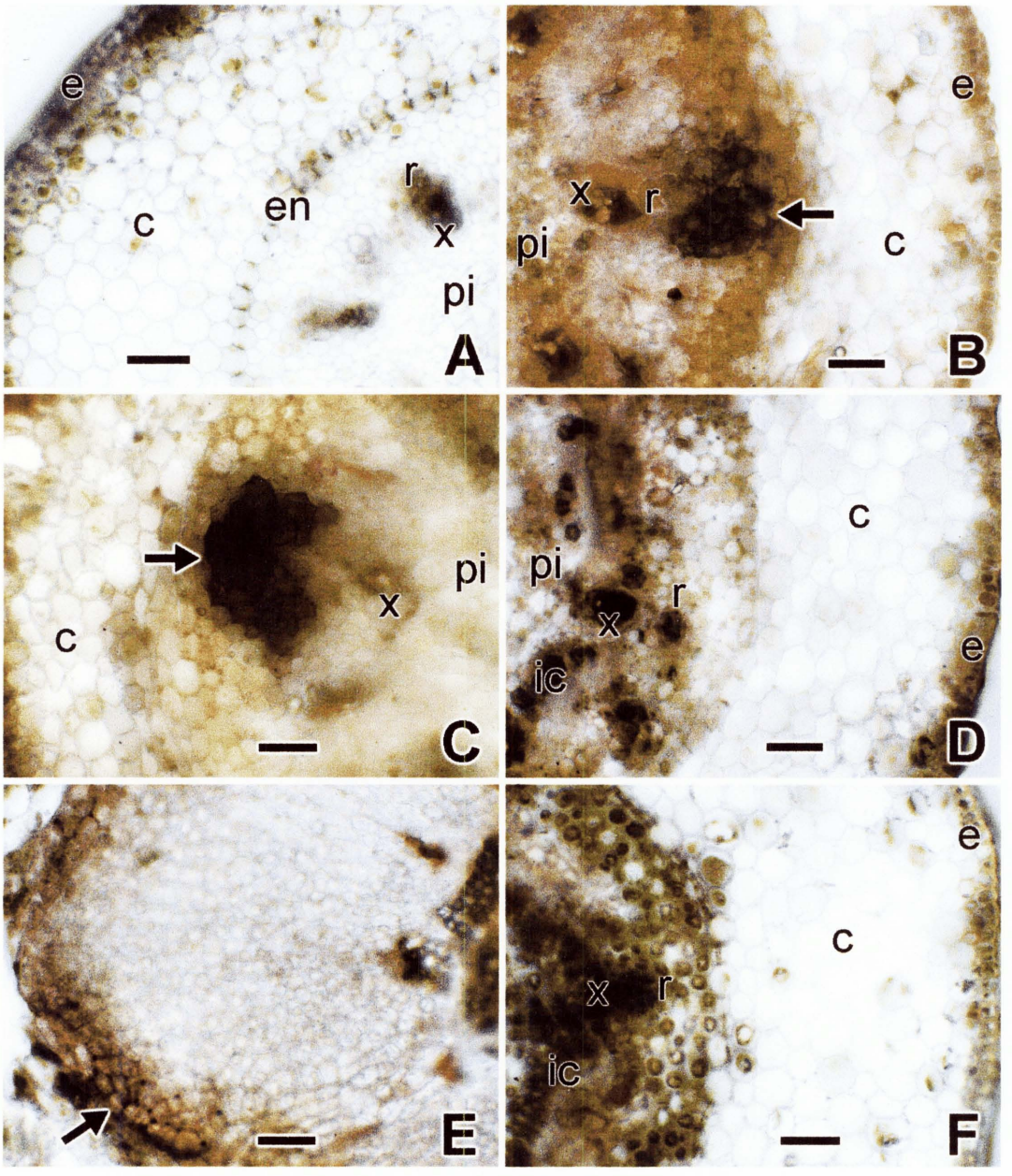

Plate 3.18 Histochemical localization of PPO activity in the basal region of the hypocotyl cuttings of *Pinus radiata*.

Black colour indicates the enzyme activity. c = Cortex, r = Differentiating resin duct, x = Protoxylem, e = Epidermis, en = endodermis, ic = interfascicular cambium. All bars = 100 μm. (A) Transverse section at day 0. (B), (C) and (D) show sections from the IBA-root-inducing treatment. (B) Transverse section at day 6. Increased PPO activity is located at meristematic locus (arrow). (C) Transverse section at day 7. Meristematic tissue indicates very strong PPO activity (arrow). (D) Transverse section of rooting-control at day 7. (E) Transverse section at day 10. PPO activity decreased and is located at the periphery of the primordium (arrow). (F) Transverse section of rooting-control at day 10.

hypocotyls of *P. radiata* at the time when cuttings were made, with only a few cells of proxylem showing SDH activity (Plate 3.19-A). The enzyme activity then increased after the excision of the hypocotyls and with the initiation of root meristematic tissues. Significant change was observed in the sections of hypocotyls treated with IBA at day 5 (Plate 3.19-B). Increased enzyme activity was located at the meristematic loci, the differentiating resin ducts and the peripheral cells. The enzyme activity continued to increase with the development of meristematic tissue in this area (Plate 3.19-C and D). It seemed that the highest enzyme activity appeared at day 7 and day 8, and then the enzyme activity decreased somewhat at day 10 (Plate 3.19-F). In contrast, the enzyme activity in rooting-control was very low during this stage, also locating at the periphery of proxylem (Plate 3.19-E).

3.8 Identification of genes expressed during rooting

One objective is to isolate the genes specifically expressed during adventitious root formation. Initially, isolation of protein markers from protein electrophoresis was expected. In this case, antibodies could be raised against purified proteins and used for screening a cDNA library. Alternatively, PCR primers could be designed based the partial protein sequencing information and subsequently PCR product could be cloned. However, the 19 identified proteins by 2D-PAGE were of such small amount that it is difficult to quantify them. Therefore, more powerful molecular technique was used.

3.8.1 Optimization of PCR

As describe in Section 2.9.3, the subtraction strategy was chosen in the present experiment. PCR was necessary to amplify the tiny amount of the subtracted ss-cDNA before cloning. Therefore, oligo d(T) was used as primer in the reaction of reverse transcription and the subsequently generated ss-cDNAs were tailed at the 3' end with dATP using terminal transferase (TdT). This allowed the amplification of the subtracted ss-cDNA by PCR using a single primer, e.g. the *Eco*RI PCR primer.

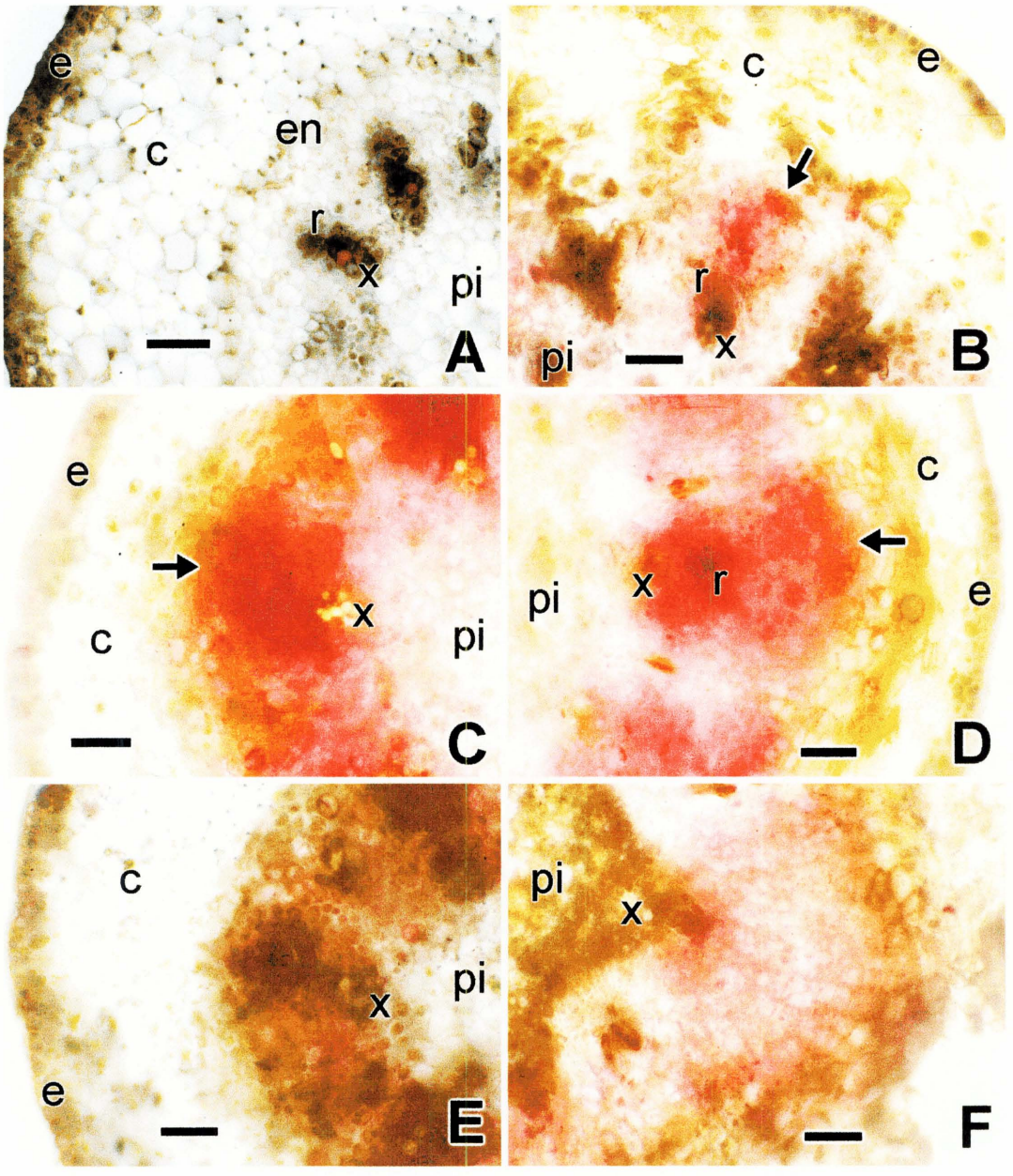

Plate 3.19 Histochemical localization of SDH activity in the basal region of the hypocotyl cuttings of *Pinus radiata*.

Purple colour indicates the enzyme activity. c = Cortex, r = Differentiating resin duct, x = Protoxylem, e = Epidermis, en = endodermis. All bars = 100 μm. (A) Transverse section at day 0. Only a few cells of proxylem showed SDH activity. (B)-(D) and (F) show sections from the IBA-root-inducing treatment. (B) Transverse section at day 5. Increased SDH activity was located at meristematic locus (arrow). (C) & (D) Transverse section at day 6 and 7, respectively. Meristematic tissue showed very strong SDH activity (arrows). (E) Transverse section of rooting-control at day 7. (F) Transverse section at day 10. Decreased SDH activity of the primordium.

Initially, the yield of the PCR product was apparently not satisfactory as only a faint, small molecular smear was present on the agarose gel (Plate 3.20 and Plate 3.21). Various concentrations of DTT, DMSO and Mg^{++}, which may increase yield or specificity (Guevara-Garcia *et al.*, 1997), were tested to improve product yield and specifity. It seemed that there was no significant improvement when DTT and DMSO were added separately (Plate 3.20), and was even worse in the case of DMSO. However, the combination of these two chemicals (at 1.5 mM) could increase the PCR yield and/or specificity (Plate 3.20, lane 8). Nevertheless, the most positive effect was found in the treatment with addition of 3 mM of $MgCl_2$ in this trial (Plate 3.20).

Therefore, different concentrations of $MgCl_2$ were then further tested to optimize the PCR. Compared to standard PCR buffer containing 1.5 mM $MgCl_2$ (*BOEHRINGER MANNHEIM*), the PCR buffer containing 2.5 mM of $MgCl_2$ significantly increased the yield and/or specificity. The PCR yield and/or specificity could be further increased in the buffer containing higher concentrations of $MgCl_2$, but there is no huge difference among the concentrations of 3.5, 4.5 and 5.5 mM (Plate 3.21). Therefore, the $MgCl_2$ concentration at 3.5 mM was chosen in the following PCR applications.

3.8.2 Cloning the PCR products

Three ratios of PCR product/vector (i.e. 1 µl/2 µl, 2 µl/2 µl, and 3 µl/2 µl) were used in order to obtain high transformation efficiency. The highest transformation efficiency was obtained in the trial of lowest ratio of PCR product/vector, while the lowest transformation efficiency was observed in the highest ratio of PCR product/vector (Table 3.3).

3.8.3 Identification of recombinant clones and insert size

Plasmid DNAs of 30 white colonies were isolated, digested by *Eco*RI and analysed on agarose gels. Inserts were observed in all of the 30 white colonies, but inserts bigger than 700 bp were not obtained (Plate 3.22 and Plate 3.23). However, the PCR product indicated that bigger than 700 bp DNA fregments were present (Plate 3.21).

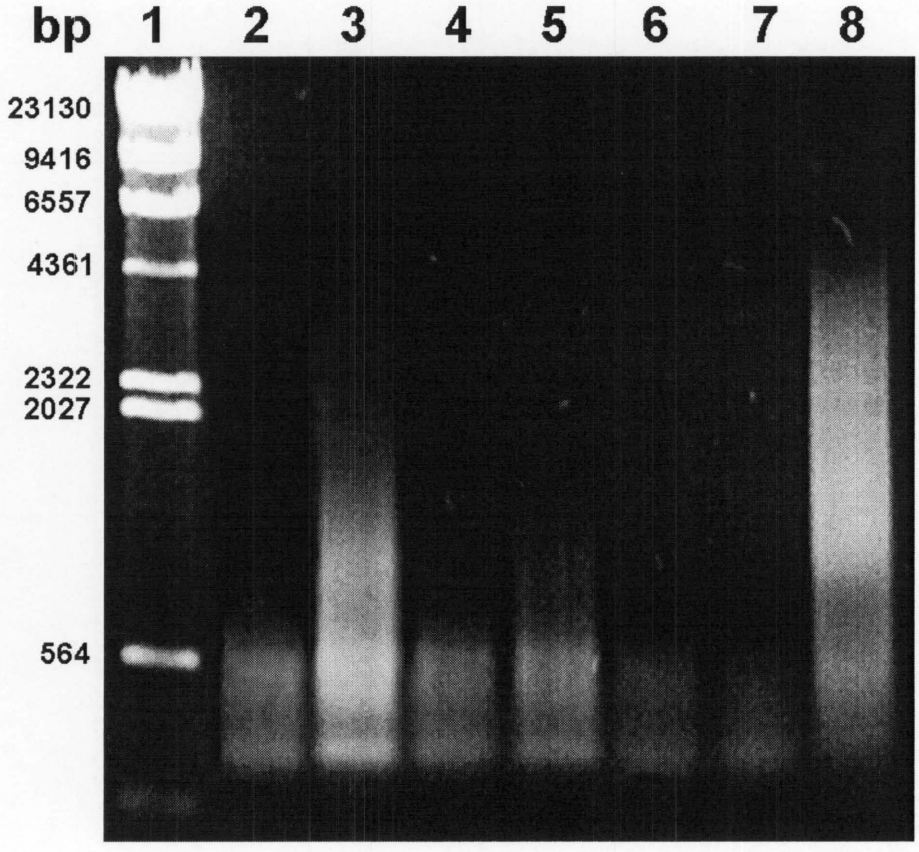

Plate 3.20 Optimization of PCR for amplification of the ss-cDNAs selected for IBA-induced adventitious root formation in de-rooted *P. radiata* hypocotyl cuttings by adding DMSO, DTT and Mg^{++}.

The PCR product was electrophoresed in a 1% agarose gel (in TAE buffer) at 8 V/cm. Lane 1: DNA molecular standards, 5 µl loaded; Lanes 2: standard PCR buffer (*BOEHRINGER MANNHEIM*, 1.5 mM Mg^{++}); Lane 3: 1.5 mM DTT + 1.5 mM DMSO; Lane 4: 1.5 mM DDT; Lane 5: 3 mM DTT; Lane 6: 1.5 mM DMSO; Lane 7: 3 mM DMSO; and Lane 8: 3 mM $MgCl_2$. 10 µl/lane loaded. Five µl of the mixture of 10 µl original tailed ss-cDNA and 25 µl the first trial of PCR was used as template DNA in each reaction.

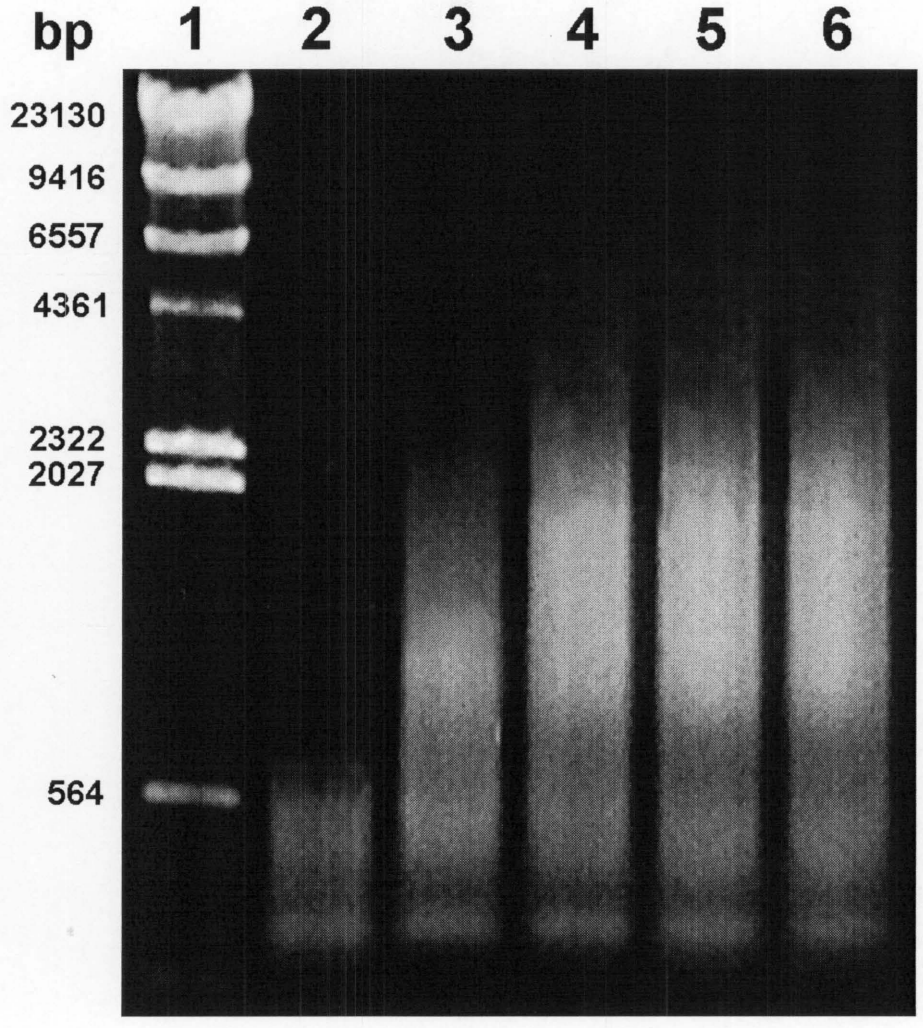

Plate 3.21 Optimization of PCR for amplification of the ss-cDNAs selected for IBA-induced adventitious root formation in de-rooted *P. radiata* hypocotyl cuttings by adding different concentrations of Mg^{++}.

The PCR product was electrophoresed in a 1% agarose gel (in TAE buffer) at 8 V/cm. Lane 1: DNA molecular standards, 5 µl loaded; Lanes 2: standard PCR buffer (*BOEHRINGER MANNHEIM*, 1.5 mM $MgCl_2$); Lane 3: 2.5 mM of $MgCl_2$; Lane 4: 3.5 mM of $MgCl_2$; Lane 5: 4.5 mM of $MgCl_2$; and Lane 6: 5.5 mM of $MgCl_2$. 10 µl/lane loaded. Two µl of the PCR product which was from the previous optimization with 3 mM $MgCl_2$ was used as template DNA in each reaction.

Table 3.3 The effects of the ratios of PCR products/vector on the ligation and transformation efficiency

Ratios of PCR products/vector (μl/μl)	Total colonies	Blue colonies	Light blue colonies	White colonies	% white colonies
1/2	299	70	131	98	32.8
2/2	221	68	86	67	30.3
3/2	138	60	41	37	26.8

3.8.4 Labelling inserts as probes

Five inserts were chosen at random for PCR-DIG labelling as probes for further hybridization. The PCR-DIG labelling method was very successful when the *Eco*RI-digested plasmid DNA acted as PCR template for the direct amplification of a large quantity of efficiently labelled vector-free probe. First, the PCR products showed very strong bands with smears on an agarose gel even though only 5 μl of the products were loaded in each lane (Plate 3.23). It is noted that the molecular weights of the labelled PCR products increased due to the incorporation of DIG-11-dUTP and the size the the primer. Second, the quantification showed that the PCR-DIG produced high yield labelled DNAs, which were similar to the DIG-labelled control DNA supplied in the DNA Labelling and Detection Kit (Plate 3.24). To find out if the un-incorporated DIG-11-dUTP might interfere with the quantification and detection of hybridization, a control that was set up from PCR mix (no cycles of DNA amplification) was performed. The result showed that most of the un-incorporated DIG-11-dUTP could be washed off the membrane. However, a trace of the un-incorporated DIG-11-dUTP could still be detected on the membrane (Plate 3.24). Therefore, purified labelled DNA was used as probe in hybridization in this study although labelled DNA could be used directly as probe without purification in most applications other than *in situ* hybridization according to the DIG/Genius (tm) User's Manual (*BOEHRINGER MANNHEIM*).

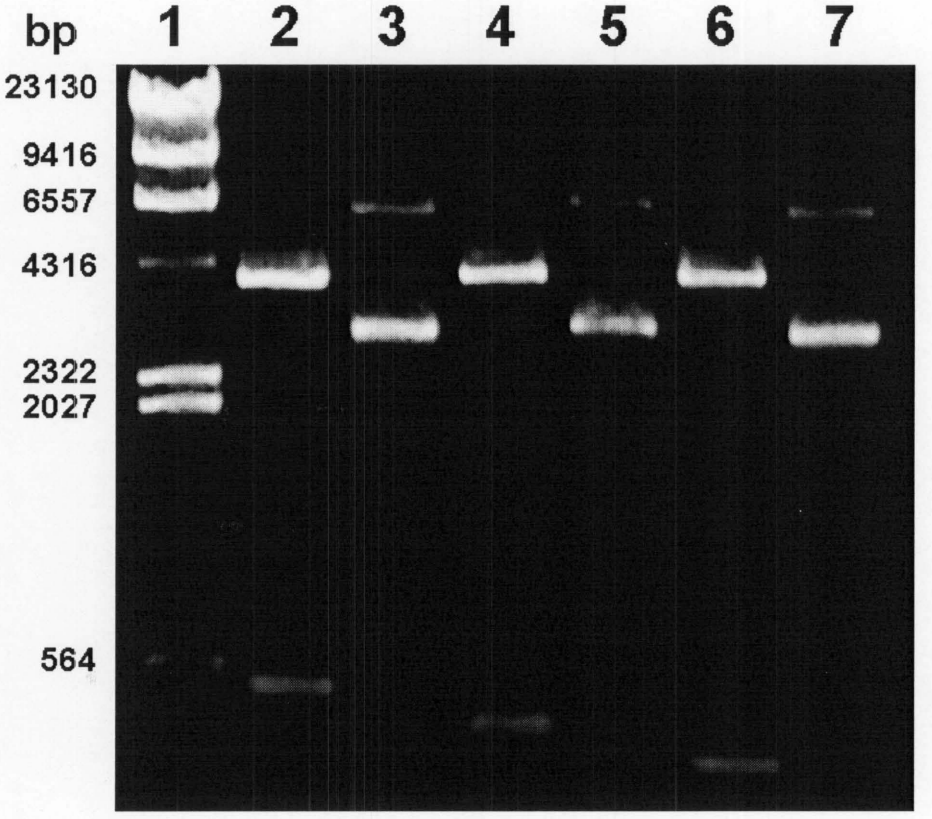

Plate 3.22 Comparison of the intact and digested plasmid DNA from three white colonies after TA cloning of the PCR products.

The PCR was performed to amplify the ss-cDNAs selected for IBA-induced adventitious root formation in de-rooted *P. radiata* hypocotyl cuttings. The DNA was electrophoresed in a 1% agarose gel (in TAE buffer) at 8 V/cm. Lane 1: DNA molecular standards, 5 µl loaded; Lanes 2, 4, and 6: digested plasmid DNA, 10 µl/lane loaded; Lanes: 3, 5, and 7: intact plasmid DNA, 10 µl/lane loaded.

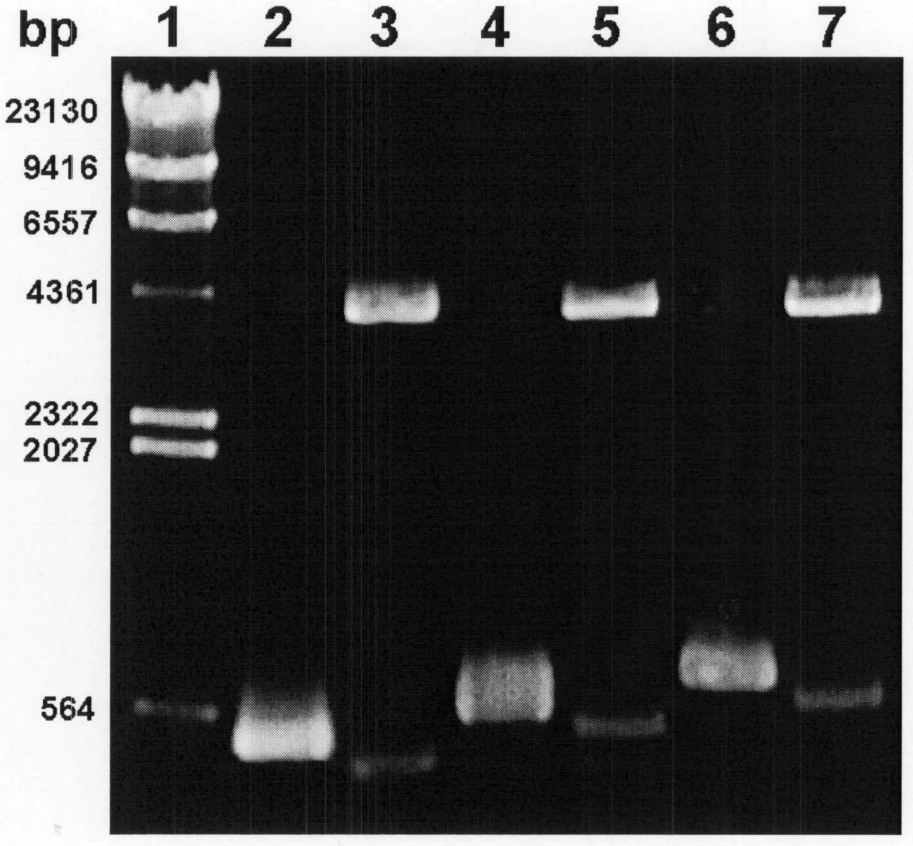

Plate 3.23 Analysis of PCR-DIG labelled DNA and digested plasmid DNA from three white colonies after TA cloning of the PCR products on an agarose gel.

The PCR was performed to amplify the ss-cDNAs selected for IBA-induced adventitious root formation in de-rooted *P. radiata* hypocotyl cuttings. The DNA was electrophoresed in a 1% agarose gel (in TAE buffer) at 8 V/cm. Lane 1: DNA molecular standards, 5 µl loaded; Lane 2, 4, and 6: inserts from three colonies were labelled by DIG-PCR during amplification, 5 µl/lane loaded; Lane 3, 5, and 7: plasmid DNAs from the three colonies were digested by *Eco*RI, 10 µl/lane loaded.

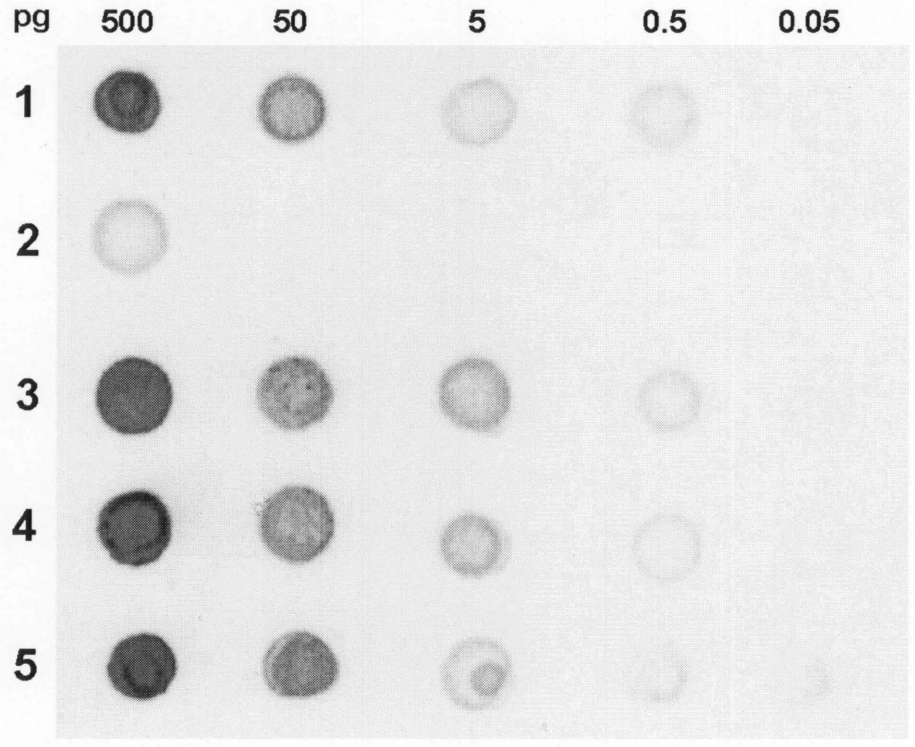

Plate 3.24 Quantification of labelled DNA by PCR-DIG.

Row 1: DIG-labelled control DNA at concentrations of 500 pg/μl, 50 pg/μl, 5 pg/μl, 0.5 pg/μl and 0.05 pg/μl (from left to right); Row 2: a control came from PCR mix (no cycles of DNA amplification); Row 3, 4 and 5: three labelled DNAs. All spots = 1 μl. All dilution schemes were the same: 1:10, 1:100, 1:1000, 1:10000, and 1:100000.

3.8.5 RNA dot blotting

The specificity of the putative rooting specific cDNAs was tested in RNA dot blotting hybridization. Equal amounts of RNA were blotted onto stripes of nylon membrane and hybridized with DIG-labeled cDNA inserts of putative rooting-specific cDNA clones. Initially, three RNA samples (day 0, day 7 from the treatments with and without IBA) were blotted on nylon membrane and hybridized with 5 probes (R1-72, R2-7, R2-22, R2-35, and R3-24).

Among the 5 clones, R1-72 appeared to represent a gene that was commonly expressed in all tissues, i.e. the expression of a 'house-keeping' gene. The signal with this probe was apparently stronger than that observed with the other probes at all three times of assays (Plate 3.25-A). With the probe R3-24, it seemed that the abundance of the transcript at day 7 from the IBA treatment was slightly higher than that at day 0 and of the non-rooting control. While with the probes of R2-7, R2-22 and R2-35, particularly R2-35, the hybridization signal was markedly enhanced in the rooting tissue at day 7 (Plate 3.25-A).

A more detailed time course study followed. Total RNA was extracted from rooting region of hypocotyls in the presence and absence of IBA at different times (day 0, 1, 4, 7, 10, and 13) and four probes (R2-7, R2-22, R2-35 and R3-24) were used. It seems that only the probes R2-7 and R2-35 were more specific to the IBA treatment. The abundance of the transcripts that are represented by these clones increased at day 4, reached their maximum at day 7 and then decreased at day 10 in the IBA treatment (Plate 3.25-B). In contrast, in the non-rooting control (without IBA in the medium) the levels of the transcripts were almost constant and low (Plate 3.25-B).

3.8.6 Southern blotting

When digested genomic DNA was probed with R2-7, there was one band in the *Bam*HI lane, three bands in *Eco*RI lane and one band in *Hin*dIII lane (Plate 3.26). The band in *Bam*HI lane was of high molecular weight (around 20 kb), the three bands in *Eco*RI lane were from 1.5 to 3 kb, and the band in *Hin*dIII lane was about 2 kb. Southern blotting analyses were also carried out using probes R2-22 and R2-35,

however, hybridization signal was not observed. This is probably due to the small sizes of these probes and the very large size of radiata pine genom (Julia Charity, personal communication).

3.9 Root induction by Agrobacterium rhizogenes

Agrobacterium rhizogenes could induce root formation in various plant species by either transfoamtion or improving the rooting environment. To test if this agrobacterium can transform radiata pine and/or improve rooting of this plant species, various methods and tissues were applied.

3.9.1 Root induction in hypocotyl tissue

3.9.1.1 Percentage of survival in different treatments

Various methods of bacterial inoculation appeared to affect the percentage survival of the cuttings or seedlings, M1 being slightly better than M2 while M3, M4 and M5 resulted in very poor survival of the cuttings or seedlings (Table 3.4). Apparently, in M1 the high survival rate was because the hypocotyl segments were cultured in a tissue culture medium. Some cuttings died because of contamination, particularly by *A. rhizogenes*, which may have come through the hypocotyl segment and grew in the medium.

In M3, M4 and M5, the low survival rates may be due to the de-rooted seedlings being unable to effectively absorb water and nutrients from the culture medium since large pieces of vermiculite form large spaces and the cut surfaces of the de-rooted seedlings could sometimes fail to be in contact with the moist vermiculite.

3.9.1.2 Rooting percentage and root number

Both *Agrobacterium rhizogenes* strains and treatment methods affected adventitious root formation in *Pinus radiata* hypocotyl tissue. High rooting percentages and root number were observed in the treatments with both strains in M1-a (with IBA in the medium) and M2, which were significantly different from their controls. Statistical

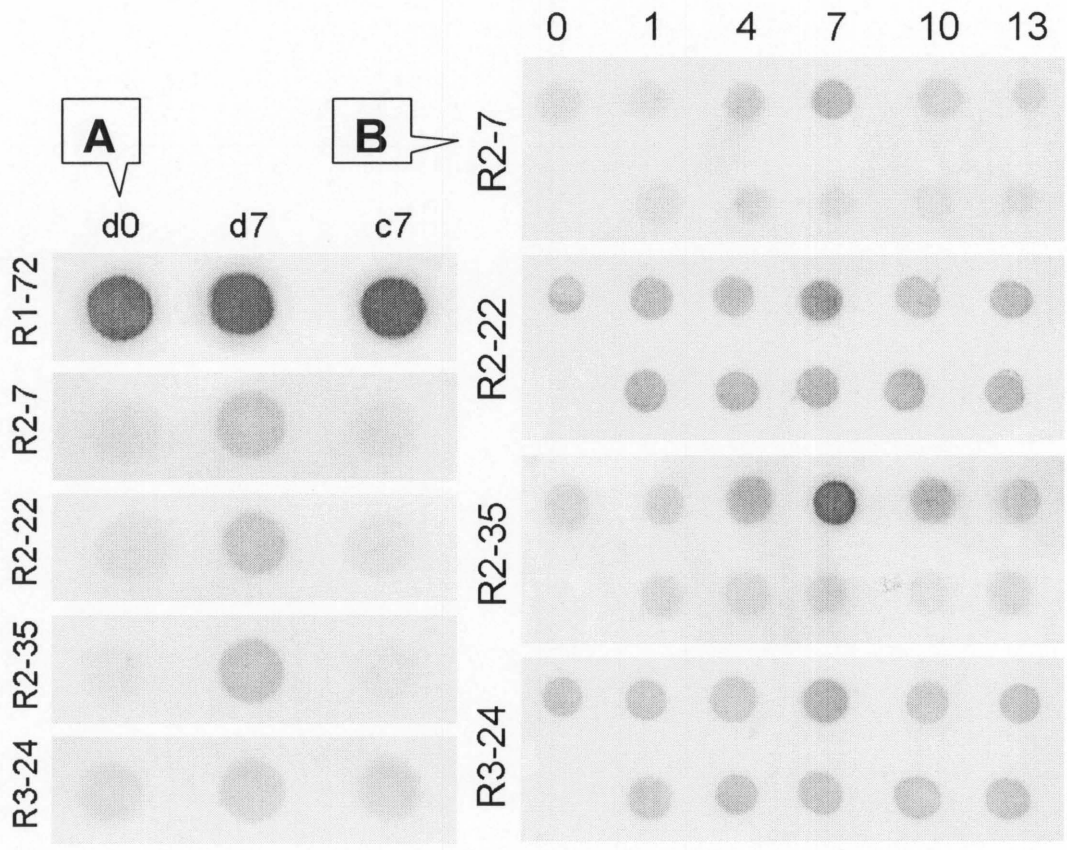

Plate 3.25 RNA dot blot analysis of total RNA extracted from the rooting region of *Pinus radiata* hypocotyls.

One μg of total RNAs were directly dotted onto each spot of the nylon membrane. (A) RNAs were extracted at day 0 (d0), day 7 from the IBA treatment (d7) or the control, i.e. without IBA in the medium (c7) as indicated on the top. Probes (R1-72, R2-7, R2-22, R2-35, and R3-24) are indicated on the left. (B) In each strip of nylon membrane, RNAs extracted from the IBA treatment and the control were dotted onto the first row and second rows, respectively. Probes and the time course of the experiment are indicated on the left and the top, respectively.

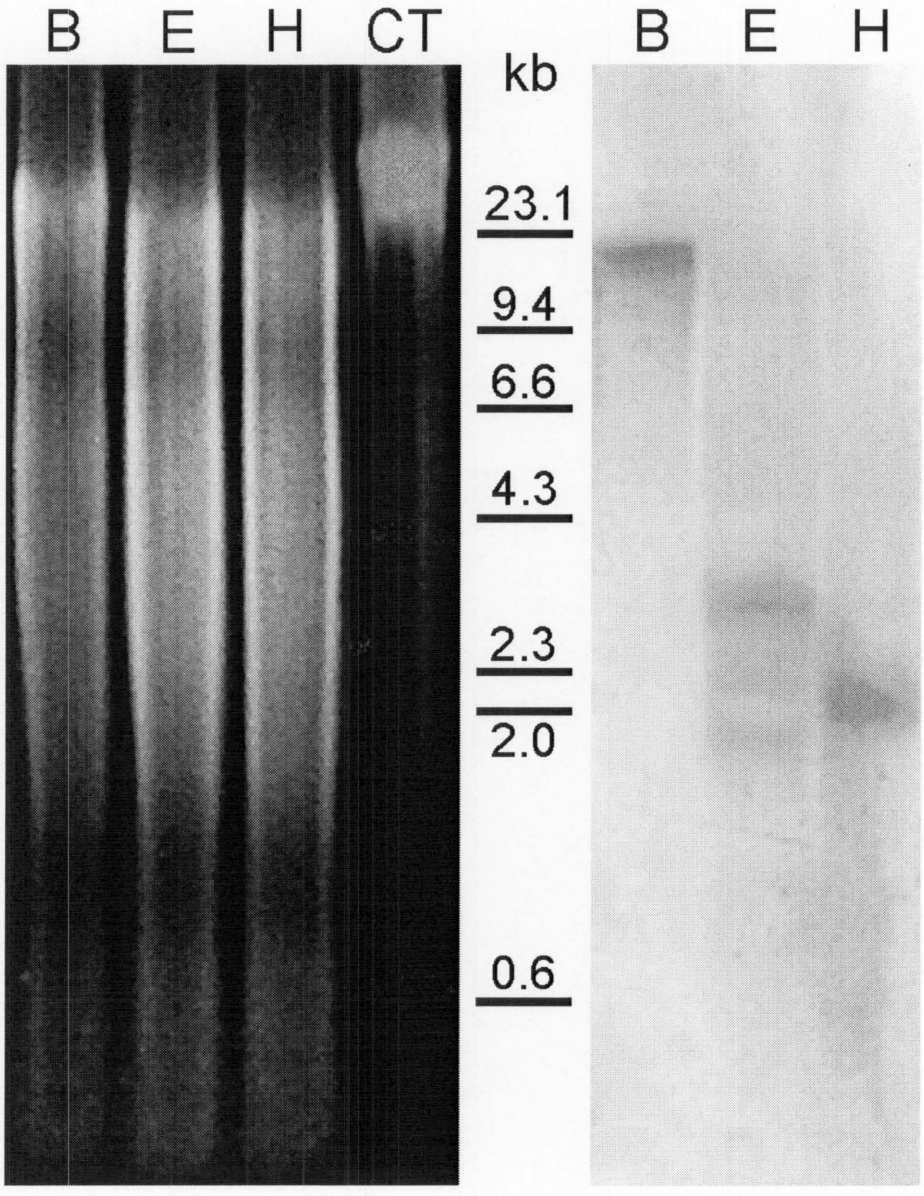

Plate 3.26 Southern blotting analysis of total *Pinus radiata* genomic DNA with the probe R2-7.

(A) Total genomic DNA was digested with *Bam*HI (lane B), *Eco*RI (lane E) and *Hind*III (lane H) and electrophoresed in a 0.7% agarose gel. Approximately 15 μg of digested or non-digested (CT) DNA was loaded in each lane. (B) The electrophoresed DNA was transferred to a nylon membrane and hybridized with the DIG-labelled probe R2-7.

Table 3.4 The results of root induction by *Agrobacterium rhizogenes* in hypocotyl tissue of *Pinus radiata*

Methods	Strains	No. of cuttings or seedlings	Survival (%)	Rooting (%)	No. of roots/cutting or seedling
M1-a	A4T	45	91.1±4.4ab	33.8±3.1cd	0.61±0.04b
	LBA9402	45	93.3±3.8ab	76.5±4.1a	1.84±0.16a
	Control	45	97.8±2.2a	0.0±0.0e	0.00±0.00c
M1-b	A4T	45	88.9±2.2ab	4.9±2.5e	0.05±0.02c
	LBA9402	45	84.4±2.2ab	18.6±3.2cde	0.29±0.05bc
	Control	45	95.6±4.4ab	0.0±0.0e	0.00±0.00c
M2	A4T	45	84.4±9.7ab	39.5±0.5bc	0.66±0.02b
	LBA9402	45	75.6±5.9bc	59.6±7.7ab	1.45±0.08a
	Control	45	77.8±5.9bc	0.0±0.0e	0.00±0.00c
M3	A4T	45	24.4±4.4d	0.0±0.0e	0.00±0.00c
	LBA9402	45	22.2±5.8d	0.0±0.0e	0.00±0.00c
	Control	45	28.9±5.8d	0.0±0.0e	0.00±0.00c
M4	A4T	45	26.7±3.8d	17.8±9.7cde	0.29±0.19bc
	LBA9402	45	20.0±3.9d	0.0±0.0e	0.00±0.00c
	Control	45	22.2±5.9d	0.0±0.0e	0.00±0.00c
M5	A4T	45	28.9±2.2d	0.0±0.0e	0.00±0.00c
	LBA9402	45	33.3±3.8cd	11.1±11.1de	0.17±0.17c
	Control	45	26.7±6.7d	0.0±0.0e	0.00±0.00c

* Mean values ± SE within a vertical column followed by the different letter are significantly different ($P<0.05$) using Tukey's method following ANOVA. Data of percentages were converted by arcsin transformation prior to ANOVA.

Data were obtained after 6 weeks from the start of bacterial inoculations.

analysis showed that root formation in the treatments of M1-a and M2 with *A. rhizogenes* was significantly different from the other treatments (except rooting percentage in M2). In M1-a, about 33.8% and 76.5% of the surviving cuttings showed regeneration of adventitious roots and every surviving cutting produced an average of 0.61 and 1.84 roots in the treatments of inoculation with A4T and LBA9402, respectively. In M2, adventitious roots formed in approximately 39.5% and 59.6% of the surviving seedlings and an average of about 0.66 and 1.45 roots were observed in each surviving seedling. Root formation was not observed in all of the controls. There was no significant difference among the treatments in M1, M3, M4 and M5 (Table 3.4).

Under a microscope, gross morphological differences were not observed between the inoculation with A4T and LBA9402 in M1-a (with IBA in the medium) and M1-b (without IBA in the medium) before day 10 after inoculation. At this stage, a bacterial film appeared on the cut surface and the cells in the area close to differentiating resin ducts, where the root primordia were initiated as described in Section 3.2, seemed to be active, suggesting the cells were dividing (Plate 3.27-A). As early as at day 14 primordia-like lumps could be observed in M1-a (Plate 3.27-B), but in M1-b the primordia were hardly seen at this stage (Plate 3.27-D). These primordia could be clearly observed at day 16 (Plate 3.27-C). Meanwhile, only calli were observed in the controls without prior bacterial inoculation (Plate 3.27-E). One week later, the roots formed in M1-a developed well as shown in Plate 3.27-F, but only enlarged primordia were observed in M1-b (Plate 3.27-G). It seemed that more calli were produced in the controls at this stage (Plate 3.27-I). At day 26, the newly formed roots developed further, and the longest root reached up to 2.3 cm (Plate 3.27-H). The data showed that addition of IBA to the medium not only increased the rooting percentage and root number, but also induced root formation earlier. In M2, the intact seedlings injected with a bacterial suspension normally formed adventitious roots from the infection site (Plate 3.28-A and B), but roots appearing from above and below the infection site were also observed in some seedlings (Plate 3.28-C). Roots formed in M4 and M5 were located at the very bottom of the cuttings (Plate 3.28-D).

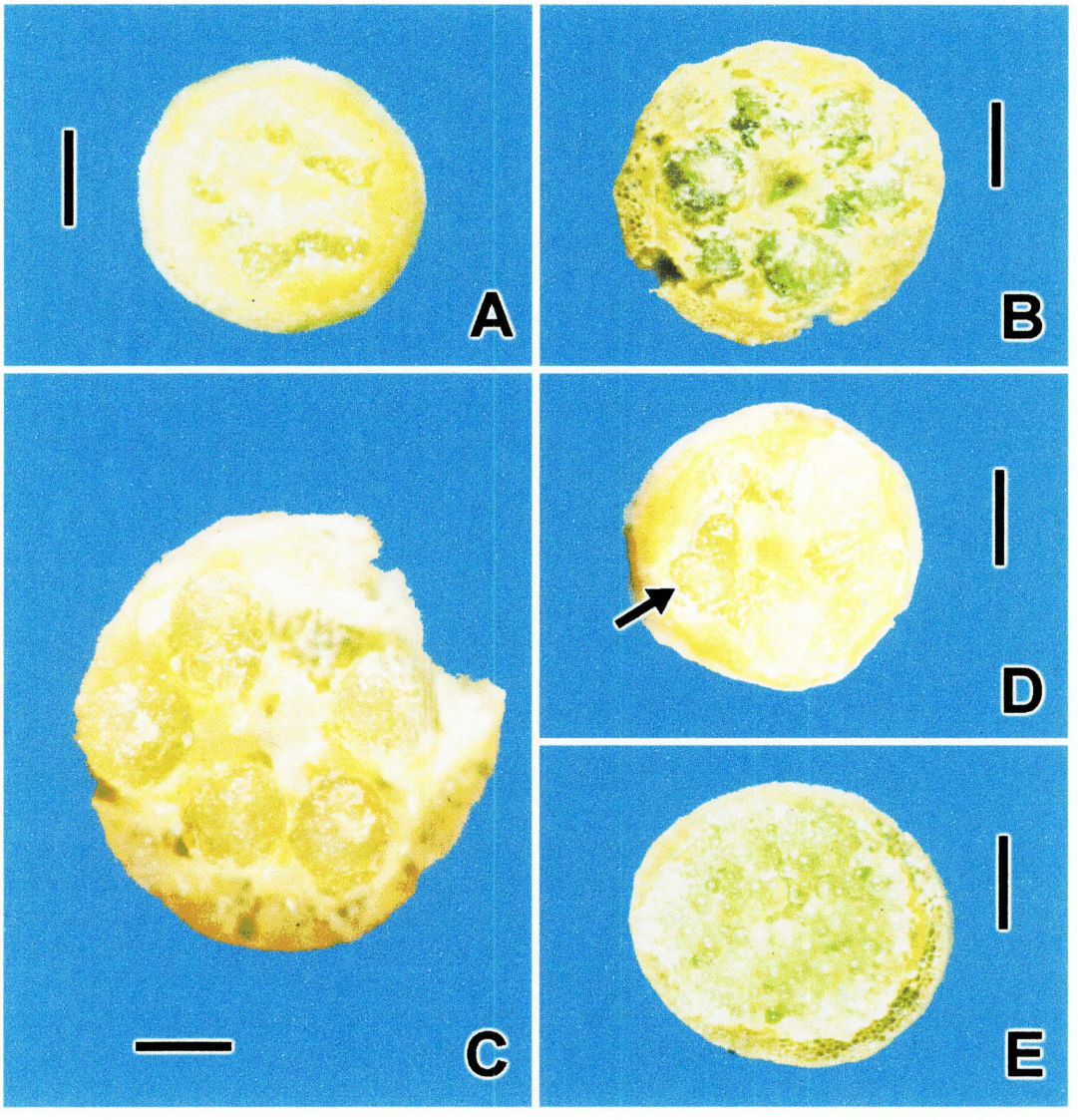

Plate 3.27 Top-view observations on root formation induced by *A. rhizogenes* in hypocotyl segments of *Pinus radiata*.

All bars = 0.5 mm (A.) A segment showing the dividing cells in the area of differentiating resin ducts at day 10 (M1-a with LBA9402). (B) & (C) Primordia-like lumps apppearing on the top of the segment inoculated with LBA9402 in M1-a at day 14 and 16, respectively. (D) A primordium (arrow) appears in M1-b with LBA at day 14. (E) Calli formed in control of M1-b at day 14.

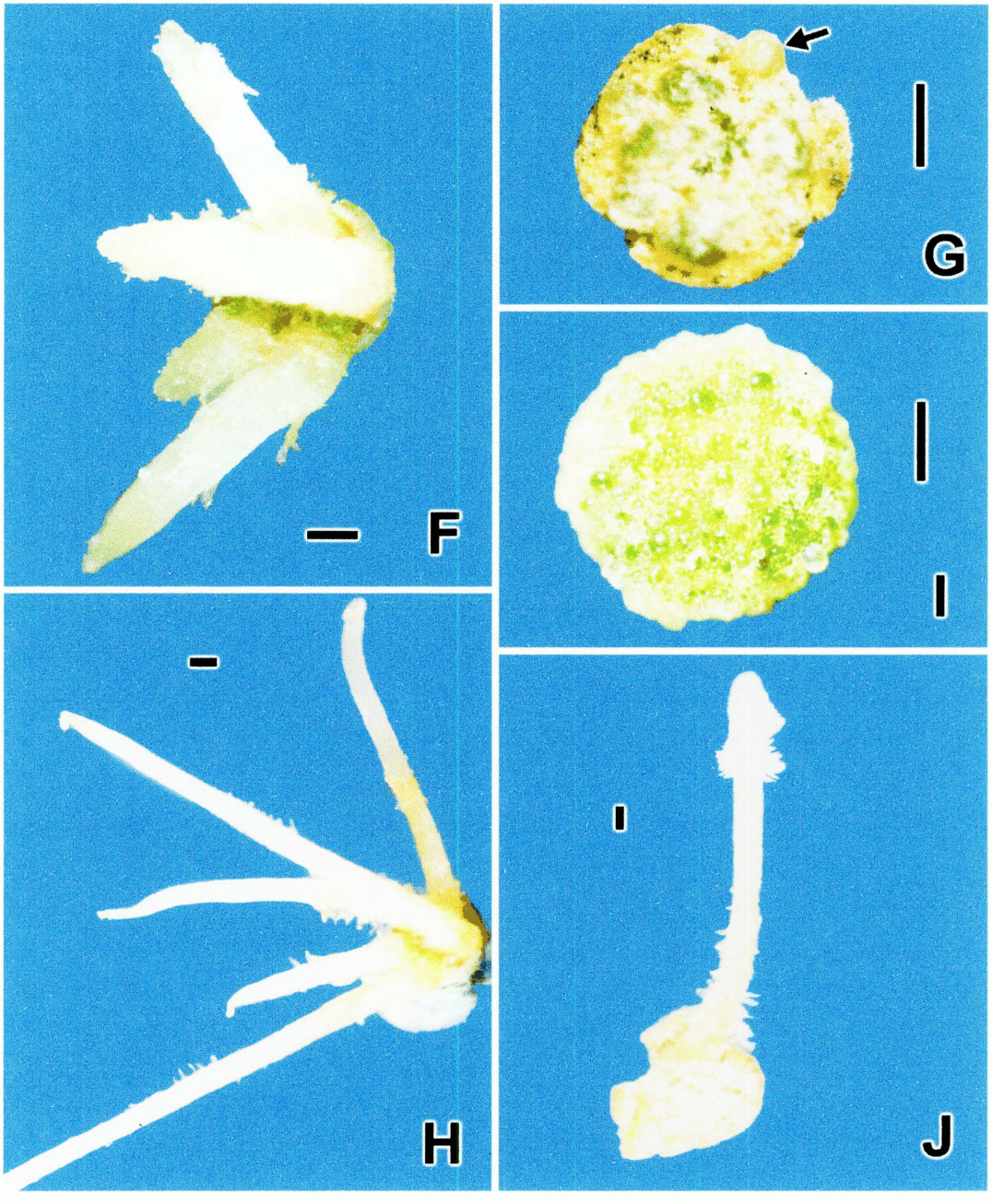

Plate 3.27 (continued) Top-view observation on root formation induced by *A. rhizogenes* in hypocotyl segments of *Pinus radiata*.

All bars = 0.5 mm (F) Adventitious roots appearing at the top of a segment in M1-a with LBA9402 at day 21. (G) A primordium (arrow) formed at the top of a segment in M1-b with LBA9402 at day 21. (H) Well developed adventitious roots in M1-a with LBA9402 at day 26. (I) Calli on the top of a segment in the control of M1-a at day 21. (J) A functional root and a small root in the treatment of M1-b with LBA9402 at day 26.

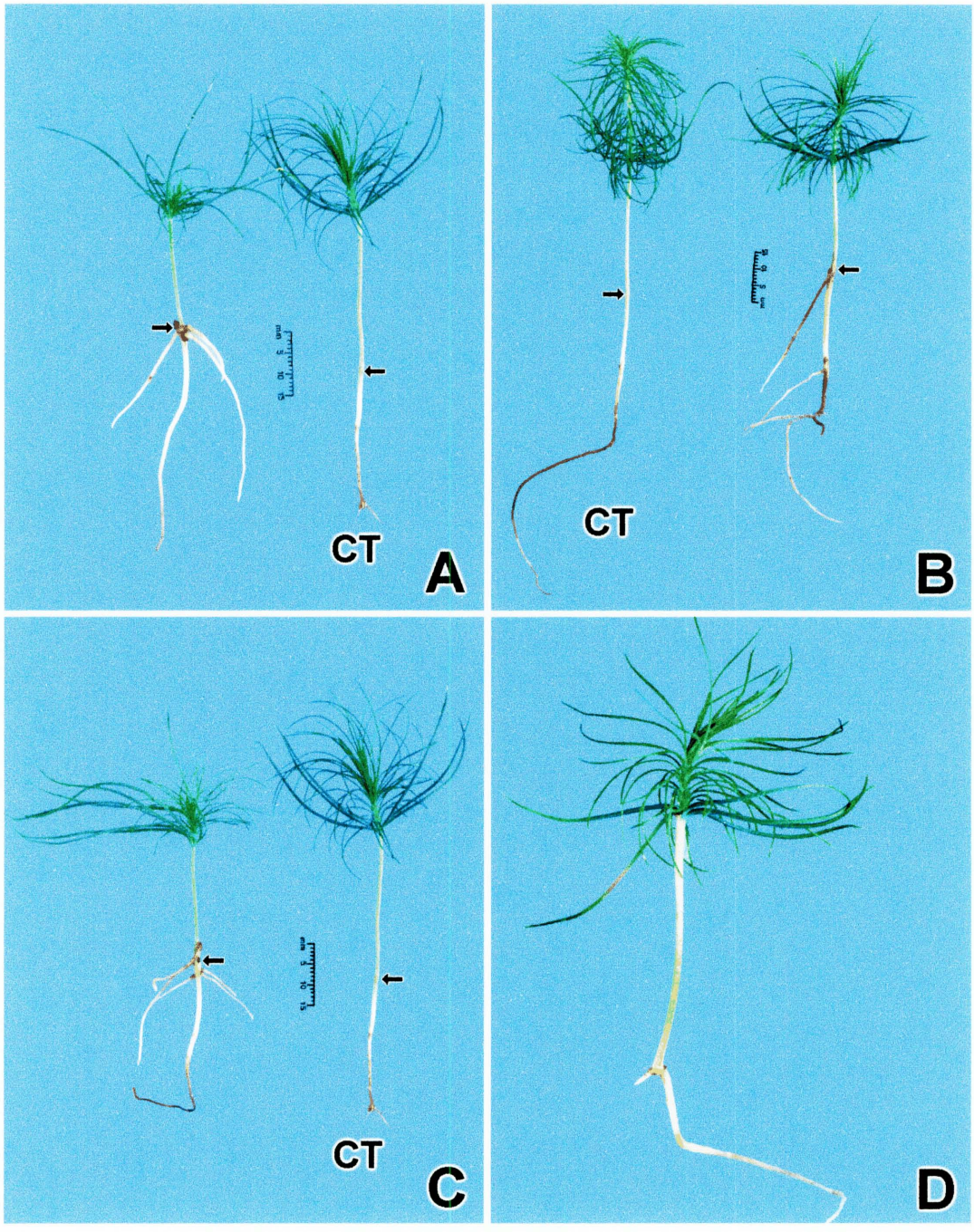

Plate 3.28 Root formation induced by *A. rhizogenes* in hypocotyls of intact seedlings (M2) and cuttings (M5).

(A) Roots induced by LBA9402 in an intact seedling and control (CT). (B) Roots induced by A4T in an intact seedling and control (CT). (C) Roots induced by LBA9402 appearing from above and below the infection site. Arrows indicate the injection sites. (D) Roots induced by LBA9402 in M5.

3.9.1.3 Effects of *A. rhizogenes* on lateral root formation

Significantly more lateral roots were found on the intact seedlings inoculated at the hypocotyls with both strains of *A. rhizogenes* (M2) compared to the non-inoculated control. On average, 3.19 and 1.56 lateral roots were recorded in the inoculations with A4T and LBA9402, respectively, while in control only an average of 0.70 lateral roots was observed (Table 3.5 and Plate 3.28-A and B). The statistical analysis showed that the differences between the two strains were significant at $p<0.05$. This means that the inoculation with *A. rhizogenes* at hypocotyl may increase lateral root number, and the strain A4T is more powerful than strain LBA9402. However, it seems that LBA9402 could induce more adventitious roots than A4T as described above.

3.9.2 Root induction in adventitious shoots

Strain LBA9402 was used to induce adventitious roots in this experiment because it seemed to work better than A4T in the previous experiment. Eight weeks after inoculation, adventitious shoots inoculated with LBA9402 rooted at 54.8%, while control shoots rooted at 8.0% (Table 3.6). Rooting percentages of the two samples were significantly different at $p<0.01$ using Chi-square test of independence. Also, the adventitious shoots inoculated with LBA9402 produced a significantly greater mean number of roots compared to its control (Table 3.6 and Plate 3.29).

Table 3.5 The effects of inoculating different strains of *A. rhizogenes* at hypocotyl region on lateral root formation in intact seedlings of *Pinus radiata*

Stains	No. of seedlings	No. of lateral roots/seedling *
A4T	45	3.19 ± 0.27 a
LBA9402	45	1.56 ± 0.17 b
Control	45	0.70 ± 0.10 c

* Mean values ± SE within the column followed by different letters are significantly different ($P<0.05$) using the Tukey's test.

Data were obtained after 6 weeks from the start of bacterial inoculations.

Table 3.6 Root induction by *A. rhizogenes* in *Pinus radiata* shoots

Treatments	No. of shoots	Rooting (%) [1]	No. of roots/shoot [2]
LBA9402	31	54.8 a	0.86 ± 0.14 a
Control	25	8.0 b	0.12 ± 0.07 b

1) Mean values within the column are significantly different at 0.01 using the Chi-square test of independence.

2) Mean values with the column are significantly different at 0.01 using t-Test.

Data were obtained after 8 weeks from the start of bacterial inoculations.

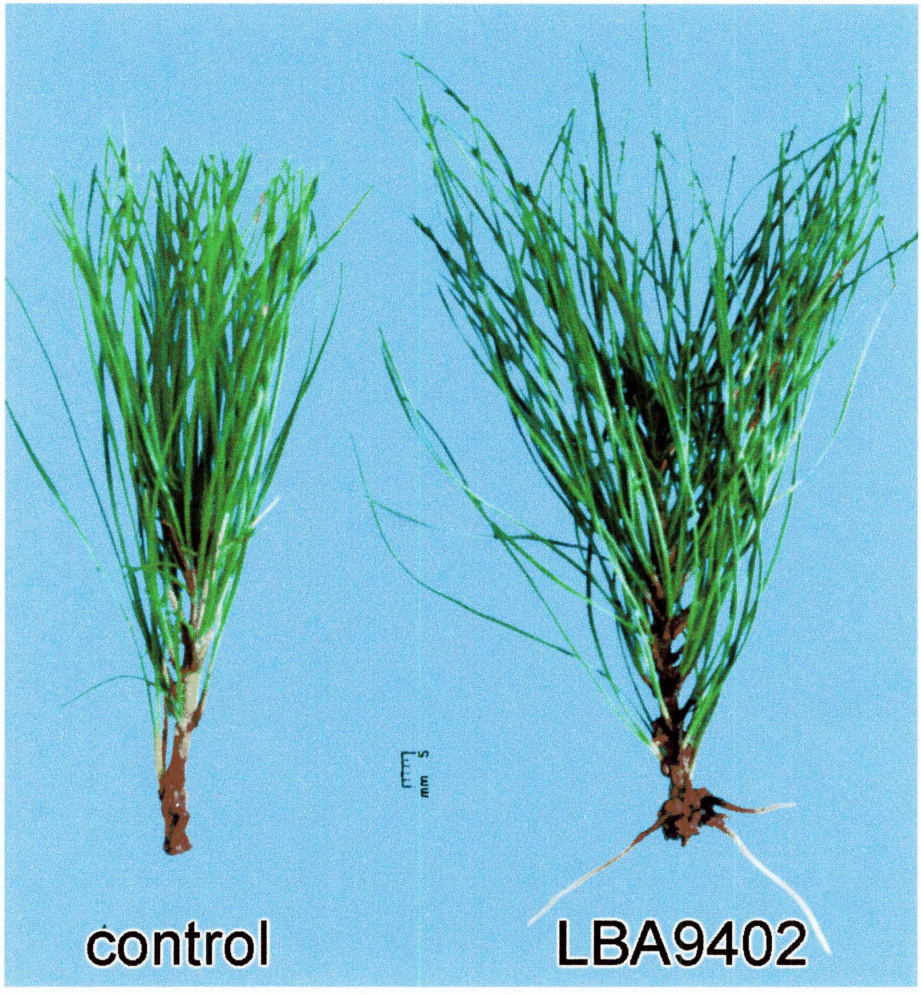

Plate 3.29 Root formation induced by *A. rhizogenes* (LBA9402) in an adventitious shoot of *Pinus radiata* at 8 weeks after infection.

The shoots were obtained from three years old greenhouse-grown seedlings.

Chapter 4
Discussion

4.1 The rooting response

Adventitious root formation in *Pinus radiata* can be induced using varying plant tissues, including hypocotyls (Smith & Thorpe, 1975b 1975a 1977), mature shoots (Field, 1934 1964; Jacobs, 1939; Mirov, 1944; Pawsey, 1950; Fielding, 1954; Sherry, 1942; Allsop, 1950; Libby & Conkle, 1966; Cameron, 1968; Thulin & Faulds, 1968), and micropropagated shoots from embryos (Reilly & Washer, 1977; Horgan & Aitken, 1981; Aitken-Christie *et al.*, 1985), seedling shoot tips (Horgan & Aitken, 1981) and mature shoots (Horgan & Holland, 1989). However, root number and rooting efficiency were largely dependent on the plant materials used. Smith & Thorpe (1975a) reported that up to 40 meristemoids (primordia), of relatively uniform size, were observed in cleared hypocotyls at day 10. Compared with hypocotyls, the other plant materials have a relatively low rooting capacity.

It is pertinent to point out the 3 considerations given for the development of the present experimental system for biochemical and molecular investigations into adventitious root initiation in radiata pine. First, many past studies were carried out under non-aseptic conditions, under which it was not possible to rule out the unpredictable interference from microbial metabolism. Second, a rooting system that produces more and uniform roots and high rooting efficiency should reduce the dilution effect caused by the cells that are not involved in rooting process. Third, to avoid studying root formation in starving or senescencing tissue, a basal nutrient medium was considered to be appropriate. Recently, De Klerk *et al.* (1999) have also

pointed out the advantages of using *in vitro* system to study adventitious root formation.

4.1.1 The effects of different media on adventitious rooting

The significance of mineral element content on adventitious root initiation and development has been recognized, although the importance of various mineral nutrients in this process is not clearly understood (Blazich, 1988; Svenson & Davies, 1995). Particularly, N, P, K, Ca, B, Mn and Zn are important for root initiation (Blazich, 1988). The results of this study indicated that adventitious root formation in hypocotyls of *P. radiata*, especially the root number on each cutting, might be influenced by the different concentrations and combinations of mineral elements and organic substances. Among these three kinds of media (GD, MS and RIM), MS has the highest ion concentration, but it could not induce the best rooting. 1/5 MS seemed too low for the best rooting response, suggesting that mineral concentration is important to rooting.

The effect of ammonium ion on morphogenesis from cultured cotyledon explants of *Panax ginseng* has been reported by Choi *et al.* (1997). In their experiment, adventitious roots were formed in hormone-free media containing little or no NH_4^+. The present study is different from that of Choi *et al.* (1997) as IBA, an effective root stimulator, was added to the medium. Nevertheless, the balance of ion concentration, combination of mineral elements and organic substances seemed to be very important.

4.1.2 The effects of sucrose concentration on adventitious rooting

Carbohydrates, the important energy pool which support adventitious root formation, could come from the storage of stock plants, photosynthesis of the cuttings during rooting, and the exogenous application. In the hypocotyls of *P. radiata*, the carbohydrates (e.g. starch) in stock plant and from photosynthesis are not enough to supply the requirement of root initiation and development. This is concluded from the evidence that root primordia formed in sucrose-free medium were less than that in media containing sucrose, and further root development from the primordia was much retarded. This indicates that sucrose in medium is not only important to root

initiation, but also to root development and growth. This is consistent with the findings in olive cuttings that sucrose treatment improved all rooting parameters: rooting efficiency (percentage), root number and root length (Wiesman & Lavee, 1995). The stimulatory effect of supplied sugars on adventitious root formation is particularly marked when cuttings are also treated with exogenous auxin (Altman & Wareing, 1975; Jarvis, 1986; Harbage *et al.*, 1993; Wiesman & Lavee, 1995). However, some investigations have indicated that sucrose applied alone did not increase the rooting capacity of cuttings and only a repeated application significantly increased the rooting of those treated with IBA (Breen & Muroaka, 1973; Breen & Muroaka, 1974; Wiesman & Lavee, 1995).

In this rooting system, it seems that 20 g/l of sucrose was the best concentration for adventitious root formation although application of more than 30 g/l of sucrose was not investigated. It has been shown that high concentrations (1-4%) of applied sucrose inhibited stock plant and root growth (Eliasson, 1978), and also inhibited enzyme activities (Lovell *et al.*, 1974; Rozema, 1979).

4.1.3 The effects of IBA treatment on adventitious root formation

Adventitious root initiation and development is largely dependent on IBA concentration and treatment time as the rooting of the cuttings in IBA-free medium was poor. It appeared that there was a compensatory effect between IBA concentration and treatment time, particularly when the IBA treatment time was less than 8 days. In a certain sense, low concentration of IBA could be compensated by relatively long IBA treatment time, and in contrast, short IBA treatment time at relative high IBA concentration might suffice. The root number was more sensitive to IBA concentration and treatment time than rooting efficiency was.

In this rooting system, it was apparent that the hypocotyl cutting of *P. radiata* treated with 9 mg/l of IBA for 10 days resulted in the best rooting response. However, Smith & Thorpe (1975b) concluded that the greatest number of meristemoids and/or primordia in hypocotyl cuttings of *P. radiata* was formed at a concentration of 3 mg/l (1.48×10^{-5} M) for 10 days. This is probably because of the different rooting systems. IBA was applied in aqueous solution in their rooting system, while IBA was added in

tissue culture medium in the present study. The IBA in the medium could be bound by organic substances and agar and therefore, the concentration of free IBA for uptake could be reduced. In addition, different pHs were used in these two rooting systems, i.e. 4.5 and 5.8, respectively. Harbage & Stimart (1996a) showed that lower concentrations of IBA were required to increase root counts as pH decreased in apple microcuttings.

The best IBA treatment time was 10 days when IBA concentration was lower than 12 mg/l. This is consistent with the result of Smith & Thorpe (1975b). This result suggests that IBA is necessary for continued growth of meristemoid and/or primordia subsequent to initiation. Such a bimodal effect of exogenous auxin in adventitious root initiation is different from that of most plants, in which auxin is only required during the inductive and/or early initiative stages (Smith & Thorpe, 1975b).

4.1.4 The effect of kinetin on adventitious rooting

A concentration of 10 mg/l of kinetin, either alone or in combination with 9 mg/l of IBA, completely inhibited adventitious root initiation. But, the morphological characters at the rooting region were different. Like control (hormone-free medium), the basal portion of the hypocotyl was not enlarged in the treatment with kinetin alone. However, in the treatment with kinetin combined with IBA enlarged basal portion was observed which was caused by proliferation of callus. Smith & Thorpe (1975b) found that a concentration of 4.64×10^{-6} M kinetin caused a pronounced reduction of root formation over the first 4 days of the experiment (root formation was nearly completely inhibited by kinetin at a concentration of 4.64×10^{-5} M). However, the kinetin treatment had no initiating effect if applied from day 6 onwards, suggesting that the prime effect of kinetin is in the inhibition of physiological events preceding the formation of the meristematic locus. Therefore, the treatment with kinetin as another rooting-control is reliable in this study, although some studies have indicated that low concentration of cytokinins is beneficial to adventitious root formation in some plant species (Kriesel, 1976; Featonby-Smith & van Staden, 1981).

4.2 Developmental sequence of adventitious rooting

This study was initiated to understand the developmental sequence of adventitious root formation in this system in order to collect samples at crucial stages. Although adventitious root formation has been induced in various plant materials in *P. radiata* for several decades, the investigation of an anatomically developmental sequence has seldom been reported, with the only one report by Smith & Thorpe (1975a). In the present rooting system, the developmental sequence of adventitious root formation was basically same as that described under non-aseptic condition (Smith & Thorpe, 1975a).

There is strong indication that the potential cells of root formation are located at the margin of a differentiating resin duct or in parenchyma tissues external to this tissue, but always within the boundary of the endodermis. Smith & Thorpe (1975a) pointed out that in *P. radiata* the formation of the root primordium begins with changes in a single cell. In the present study, it is not clear whether that the root primordia arise from a single cell or from a group of cells. However, several activated cells were frequently observed around each potential site of root initiation. This happened as early as around day 4, with a few adjacent cells beginning to divide. Furthermore, the findings of two meristematic clusters occurring side by side at day 7 onwards further support this suggestion.

Different stages in root formation have been observed by many authors (Argles, 1959; Girouard, 1967; Cameron & Thomson, 1969; Mitsuhashi-Kato *et al.*, 1978b 1978a; Druart *et al.*, 1982; Hartmann & Kester, 1983; White & Lovell, 1984; De Klerk *et al.*, 1995), but there has been no agreement as to the number and the nature of the stages and also in the terminology used (Lovell & White, 1986). Because of the similar results to that of Smith & Thorpe (1975a), their definition has been directly adopted in this study. Adventitious root formation in hypocotyls of *P. radiata* comprises three distinct stages: (1) the pre-initiative phase (before day 4), this period preceding formation of meristematic locus; (2) the initiative phase (day 4 to 10), the initiation of meristematic locus and then continued division of the meristematic cells to form primordium; (3) post-initiative phase (day 10 to 13), further development of the primordium into an effective root and emergence.

4.3 Fresh weight

It is expected that the fresh and dry weight of the rooting region would be increased in the rooting treatment during the time course. This is because of the continued division of the meristematic tissue, which subsequently led to the formation of primordia. However, dry weight was not estimated as the rooting region was extremely small and a huge number of cuttings was required. Fresh weight also increased markedly in the treatment with IBA+kinetin, in which callus was formed, at day 7 onwards. In control and the treatment with kinetin alone, fresh weight increased slightly during the time course.

4.4 Protein changes

It has been reported that auxin-induced formation of adventitious roots is associated with an increase in general soluble proteins (Kantharaj *et al.*, 1979) and some specific proteins (Dhindsa *et al.*, 1987; Oliver *et al.*, 1994; Kang, 1995). Therefore, protein contents were estimated and the proteins were analysed by 1D SDS-PAGE and 2D PAGE. The aims were to explore whether adventitious root formation in *P. radiata* is correlated with molecular events that might be used as markers for the various stages of the rooting process.

4.4.1 Protein content and adventitious rooting

An analysis of the amount of buffer soluble proteins showed that the protein content dropped in all treatments during the first day (Fig. 4). This pattern is similar to the result in a study of protein changes associated with auxin-stimulated and kinetin-inhibited lateral root initiation in lettuce roots (Macisaac & Sawhney, 1990). This is possibly due to the uptake of water in the cuttings. The decrease continued till the end of time course (day 13) in control, treatments with IBA+kinetin and kinetin alone. In contrast, the protein content in IBA treatment increased to its maximum till day 7 and then decreased. The increase before day 4 could be attributed to some pre-morphological events in the rooting process. However, the increase at day 4-7 coincided with the onset of cell division and formation of sphere of meristematic tissue. The decrease of proteins may be ascribed to the root development as the

protein content in roots is lowest among cotyledons, hypocotyls and roots (data not shown). The result clearly indicates that the IBA-induced adventitious root initiation (at day 7) in the cuttings of *Pinus radiata* hypocotyls is associated with an increase of buffer soluble proteins because increases of protein content were not detected in controls including the treatments with IBA+kinetin and kinetin alone. This has also been observed during lateral root initiation in *Lactuca sativa* (Macisaac & Sawhney, 1990) and during adventitious root initiation in *Hydrangea macrophylla* (Molnar & LaCroix, 1972a).

4.4.2 1D SDS-PAGE analysis of proteins

Storage proteins have been used as markers for somatic embryogenesis in *Brassica napus* (Crouch & Bopp, 1984) and *Trifolium* (McGee *et al.*, 1989), while other proteins have been used to identify the time of bud regeneration in excised cotyledons of *P. radiata* (Villalobos *et al.*, 1984) and as markers to outline stages in axillary bud development in *Pisum sativum* (Stafstrom & Sussex, 1988). Stage specific proteins in cultured melon cotyledons can also be used to distinguish between the process of root formation and shoot formation, prior to the formation of an identifiable structure (Leshem & Sussex, 1990).

It has been reported that the auxin-induced onset of cell division in the rooting process was accompanied by some specific proteins (Dhindsa *et al.*, 1987; Kantharaj & Padmanabhan, 1990; Macisaac & Sawhney, 1990; Oliver *et al.*, 1994; Kang, 1995). As shown by 1D SDS-PAGE, several polypeptides were specifically present at some stages during auxin-induced lateral root formation in *Lactuca sativa* (Macisaac & Sawhney, 1990) and adventitious root formation in *Vigna radiata* (Dhindsa *et al.*, 1987). However, in some cases the rooting-specific proteins could not be detected by one-dimensional electrophoresis. For example, Kang *et al* (1995) could not detect any protein that was associated with root formation by 1D SDS-PAGE, but 15 proteins associated with the process were observed by 2D-PAGE.

In this study, rooting-specific protein was not detected by the comparing the protein bands in the rooting inducing treatment with IBA and the other 3 non-root forming conditions, probably due to resolution limitations of 1D SDS-PAGE.

However, the changes of protein patterns in response to other events such as wounding and tissue culturing were observed.

4.4.3 2D SDS-PAGE analysis of proteins

Variation among sample extracts, 1D and 2D electrophoresis, and staining, no matter how stringently a protocol is adhered to, can result in subtle variations between gels (Dunbar, 1987). The procedure used in the 2D separation of proteins in this study combined many of the latest modifications to O'Farrell's original protocol (O'Farrell, 1975), to increase gel resolution and reproducibility (Duncan & Hershey, 1984; Mayer *et al.*, 1987; Hochstrasser *et al.*, 1988a 1988b). Despite this, many inconclusive changes in protein patterns were observed as a result of the various treatments of radiata pine hypocotyl cuttings.

The present study of 2D SDS-PAGE analysis of proteins demonstrated that there are many marked changes in the protein patterns associated with the excision of hypocotyls and IBA-induced adventitious root formation in *P. radiata*. Preliminary comparison indicated that there were some changes in the patterns of the proteins with molecular weights lower than 45 kDa and it was hard to discern different protein patterns between the treatment with IBA (rooting) and control (non-rooting) above this region. Therefore, the changes of protein patterns between these two treatments at different times were carefully studied in this area below 45 kDa, where 336 individual proteins were detected during the time course, including 290 proteins that were observed at day 0 and 46 proteins that were recorded as newly detected ones after the excision of hypocotyls.

Among the 290 proteins that were present at day 0, there were 108 proteins that were of the products of so-called "housekeeping-genes" which are likely to involve basic metabolism because this kind of protein was detected at an approximately constant level throughout the time course in all hypocotyls irrespective of the treatment.

There were 134 proteins that disappeared or decreased in the treatments with or without IBA during the time course. The role of these proteins is unclear, but it is suggested that they may be related to hypocotyl physiology and/or autotrophic growth.

Fifteen of the 134 proteins were of day 0 specific proteins which appeared at day 0 only (located by ✧). It is noted that all of these proteins were located in the basic and neutral area, i.e. none of them was of acid. In this group, it seems that the addition of IBA could influence the disappearing behaviours of some proteins, because there were 30 proteins that disappeared firstly in control, but another 18 proteins disappeared firstly in the hypocotyls treated with IBA. It is noted that there were 60 proteins that disappeared irregularly at some stages during the time course, for example No.166 (Table 3.2 and Appendix B), in IBA treatment, disappeared at day 1-4, appeared at day 7-10, then disappeared at day 13 again, while in control it was present at day 7 but disappeared at all other times. It is not clear whether the irregular behaviours of the proteins were due to variation or due to unknown biochemical and physiological roles these proteins played during the process.

There are two particular groups of proteins that were present at day 0 but disappeared in the treatment with IBA only and disappeared in control only, respectively. It is tentatively proposed that the accumulation of the former group of these proteins was inhibited by IBA. The role of the latter group is unclear but it may be related to original root growth.

Another group of proteins was specifically present in control only (No. 75, 98, 106, 107, 108, and 109, Table 3.2 and Appendix B). These proteins may be related to hypocotyl growth or wounding, but inhibited by IBA in the treatment with IBA. However, whether these proteins are involved in hypocotyl physiology or wounding is not known at this point.

4.4.3.1 Proteins associated with wounding and/or tissue culture

The 21 proteins (located in square □ in Plate 3.6-Plate 3.15) that were newly present at both treatments with IBA and control are obviously associated with the response of the hypocotyls to de-rooting: wounding and/or tissue culture. Dhindsa *et al.* (1987) pointed out that since wounding is known to involve the biosynthesis and release of ethylene, some of these proteins may be similar to the reported ethylene-induced proteins (Zurfluh & Guilfoyle, 1982; Nichols & Laties, 1984). Particularly, No. 39 and 101 were detectable at day 1 and increased to their maximum in abundance at day

7 and then their levels kept constant. Meanwhile, No. 40 was detectable at day 4 and increased to its maximum in abundance at day 10 and then its level kept relatively constant.

Some authors have concluded that wounding plays an important role in adventitious root formation in some species (Poapst & Durkee, 1967; Bassuk & Howard, 1981). Wounding, as mentioned above, is involved in the biosynthesis and release of ethylene, which may promote the formation of adventitious roots. The activity of some enzymes such as PO and PPO can also be enhanced by wounding. Haissig (1974a) pointed out that both wounding and IAA treatment can enhance PO activity.

It is therefore possible that wounding-induced ethylene could mediate increases in some enzyme activities during adventitious root formation. PPO activity can also be enhanced by wounding (Haissig, 1974a). Cell damage by wounding allows PPO and substrates to come into contact, thereby facilitating the oxidation of phenolics (Hand, 1994), which may form cofactors necessary for rooting (Haissig, 1974a; Bhattacharya, 1988).

4.4.3.2 Proteins associated with root formation

The most important finding of this investigation, in terms of the major objective of the study, is that IBA treatment (rooting) causes specific proteins to be observed although most of these proteins seem to be present in low abundance. Among the 19 proteins in this group, 4 proteins (No. 38, 202, 203, 326, marked in △ in Plate 3.6, Plate 3.8, Plate 3.10 and Plate 3.12) that were observed at day 1 after the excision of hypocotyls may be induced by IBA *per se* and not be involved in root formation as histological investigation showed that the first cell division happened around day 4. No. 202 and 203 are located at the very basic end during IEF electrophoresis, therefore, are not reliable.

There are 4 proteins (No. 42, 328, 329, and 333, marked in ◇ in Plate 3.6 and Plate 3.8) that were observed in the treatment with IBA only during day 4-7. The nature of these proteins is not known, but the timing of their presence coincides with the onset of cell division at the margin of the differentiating resin ducts or in

parenchyma tissue external to this tissue, where the root primordia are initiated. The induction of meristematic activity involves structural alteration, i.e. dedifferentiation and formation of meristematic tissue. Therefore, these proteins could be involved in these processes.

It seems that there is an alteration in gene expression in hypocotyls of *P. radiata* associated with adventitious root formation. The accumulation of another 7 proteins (marked in O in Plate 3.10, Plate 3.12 and Plate 3.14) is associated with root primordium formation and development. No. 213 and 334 were present during day 7-10, while No. 104, 105, 211, 212 and 335 were observed during day 7-13. These proteins might be involved with primordium formation and/or root development.

There are 2 proteins (No. 43 and 336, indicated by ◄ in Plate 3.12 and Plate 3.14) that were present during day 10-13 and could be associated with root development. Interestingly, there are two other proteins (No.41 and 103, indicated by →) seems to be associated with the whole process of root formation as they could be observed during day 4-13.

However, the changes of these proteins reported here are of very small magnitude. This is not surprising. Unlike the wound response where all of the hypocotyl cells close to cut-surface are likely to be affected, only a small fraction of the cells of the hypocotyls is involved in these processes and so their representation in the overall protein patterns is expected to be diluted.

4.5 Changes of carbohydrate content

Carbohydrates are obviously important to adventitious root formation because they serve as an energy source and yield the carbon skeletons needed for the production of new tissues, although the relationship between carbohydrates and adventitious root formation has remained controversial for several decades (Veierskov, 1988). Based on the available data, Libby & Conkle (1966) suggested that in *P. radiata* the rooting ability depends, to a large extent, upon the amount of food (carbohydrates, proteins, etc.) reserves present at the time when cuttings are made.

4.5.1 Free sugar

Despite of different levels and time sequences, changes of free sugar patterns between the treatments with IBA and IBA+kinetin were similar to each other, but different from the other two treatments. Nevertheless, root formation did not take place in treatments other than with IBA alone. This means that IBA can not only induce root formation but also affect some other physiological or biochemical processes. It is noted that there was a marked drop in the sugar content at day 7 in the treatment with IBA. Sugar was probably consumed during the formation of meristematic tissue. The increase of free sugar content after the drop may be due to the starch hydrolysis as the starch content decreased after day 7. The fact that sugar content was higher in IBA than in the other 3 treatments throughout the process of root formation implies somehow changes in sugars were associated with root formation (Figure 3.5).

Sugar content increased in hypocotyls that were cultured in the sucrose-containing medium during the first day, whereas it decreased a little in the sucrose-free medium. This suggests that the increase of free sugars in the sucrose medium during the first day may be due to the uptake of sucrose from the medium after the cuttings were suddenly exposed to sucrose medium. When the cuttings were inserted into the sucrose-free medium, the sugar in the hypocotyls might be consumed for growth, or even basipetally lost to the medium. Therefore, the level of sugar content dropped. However, IBA may regulate the metabolism of the cuttings, e.g. respiratory rate may especially increase in the auxin-treated cuttings (Strydom & Hartmann, 1960). This may be accompanied by someway of stimulating the increase of sugar content as substrates although root primordia failed to develop into functional roots in the absence of sucrose supply from the medium. This is supported by the evidence that carbohydrate concentrations in cuttings may be influenced by auxin treatment, which can enhance mobilization of carbohydrate from leaves and upper stem, and increase transport to rooting zone (see review by Haissig, 1986 and references therein). Webster & Van't Hof (1970) also reported that the provision of sucrose increased the rates of RNA and protein synthesis which were prerequisites for cell division and DNA synthesis. In contrast, the sugar content remained at a much lower level in hypocotyls cultured in the sucrose-free medium without IBA.

4.5.2 Starch content

The accumulation of starch in tissues under rooting conditions has been reported in a number of studies (Molnar & LaCroix, 1972b; Coleman & Greyson, 1977; Burritt, 1992). The deposition of starch occurs prior to the formation of meristematic tissues and often prior to any observable cell division.

Investigation into starch content in the basal hypocotyls (rooting region) indicated that the significant peak of starch content in the treatment with IBA was not observed in the other treatments, suggesting that starch accumulation in rooting part could have potential as a biochemical marker in at least radiata pine.

To determine whether this character is linked to rooting process, starch content in the non-rooting portion of the hypocotyls was estimated (Figure 3.8). The result indicated that the starch content in the treatment with IBA was not different from that in the control (growth regulator-free medium). Nevertheless, the pattern of the starch content in the treatment with IBA+kinetin appeared to contrast with that of kinetin alone. This was probably caused by some unknown effect of kinetin.

Starch content in hypocotyls cultured in sucrose-free medium was measured in order to examine whether sucrose in the medium contributed to the starch accumulation. It increased during the first 7 days in the treatment with IBA, in which root primordia were formed but failed to further develop into functional roots, then decreased onward (Figure 3.9). This decrease could be ascribed to it being consumed during root initiation and development. While the starch content in the controls (both IBA- and sucrose-free) increased rather gradually during the whole time course. Statistical analysis (one way ANOVA followed by Tukey's multiple comparison, $P<0.05$) showed that the starch contents in control at different times were not significantly different until day 10. However, the increase of starch content in the treatment with IBA was statistically significant, particularly at day 7 it was significantly different from all other assay times. A further determination at $P<0.01$ was performed, showing that the starch content of IBA treatment at day 7 was not different from that at day 4 and 10, but was significantly different from all other assay times. It is noted that the peak of starch content in this case was delayed till day 7 rather than day 4 as in the sucrose medium.

Therefore, it appeared that the starch accumulation in the rooting region was associated with root initiation and development, and that the sucrose in the medium could contribute to this process. It is assumed that starch content in the hypocotyls cultured in the sucrose medium was enough to support the requirement for root initiation and development, but it was insufficient to support further root development in the treatment with IBA minus sucrose in the medium.

The starch content in the rooting region of the hypocotyls cultured in the sucrose medium was much higher than that in the non-rooting region of the same hypocotyls and in the rooting region of the hypocotyls cultured in sucrose-free medium. This evidence may indicate that the starch grains in the rooting region could partially come from sucrose in the medium, if it is available in the medium, and that starch accumulation was associated with adventitious root formation. The possible hypothesis is that the induction of root formation promotes starch deposition and then it is consumed as energy and carbon skeletons for root development (Haissig, 1974b, 1986; De Klerk, 1996; Jásik & De Klerk, 1997). This assumption is supported by recent histological investigation in apple stem disks by Jásik & De Klerk (1997) and the starch histological localization in this study.

Histochemical investigation of starch localization was consistent with the starch determination in the extracts of the hypocotyls. Starch grains were hardly observed in the rooting region at day 0 when the cuttings were made. However, the quantity of starch grains increased and reached maximum in the treatment with IBA during day 4-7, while in control only few grains were observed. Similar results were obtained in *Hydrangea macrophylla* (Molnar & LaCroix, 1972b).

4.6 Enzyme changes and root formation

4.6.1 Peroxidase (PO)

Colorimetric assays of tissue extracts showed that PO activity in all cases increased at the beginning and then remained relatively constant until the end of the process, and that the enzyme activity was not significantly different among the four treatments (Plate 3.17). This kind of pattern has also been found in other rooted plant tissues,

e.g. avocado microcuttings (García-Gómez *et al.*, 1995), hazelnut cotyledons (González *et al.*, 1991), *Phaseolus vulgaris* (Ben-Efraim *et al.*, 1990) and *Salix tetrasperma* (Bhattacharya *et al.*, 1978). An increase of PO activity was expected at the beginning in all cases because PO was likely to regulate auxin levels during rooting process and could be triggered by wounding (excision) to cope with any increase in toxic H_2O_2 resulting from a high respiratory activity in response to wounding. PO reportedly seems to be the most ubiquitous and effective system for H_2O_2 elimination in plant cells (Cano *et al.*, 1996).

From the enzyme assay results, the relationship between PO activity and root formation appeared ambiguous as there was a similar pattern of PO activity in control, IBA, IBA+kinetin and kinetin. Furthermore, in the IBA treatment, the histochemical study showed that at day 4 increased enzyme activity was observed in almost all tissues. Epidermis and pith, particularly the meristematic loci gave relatively strong reaction, with somewhat lower activity in the cortex. With the initiation of root primordia, increasing PO activity could be detected at the root primordia and developing roots. Histochemical investigations in other plants also obtained similar results (Molnar & LaCroix, 1972b; García-Gómez *et al.*, 1995). However, this is not consistent with the data obtained by the colorimetric assay of tissue extracts. This may be because in the case of colorimetric assay of tissue extract only buffer-soluble PO was assayed, but the activities of all kinds of PO, including buffer soluble, ionically and covalently bound to cell wall could have been assayed in the case of histochemical study.

The histological study revealed a close association of PO activity with root primordial initiation and development. POs are involved in cell wall genesis; it catalyses the formation of diferulate (Lamport, 1986; Fry, 1987b) and isodityrosine cross-links of extensins (Fry, 1987a). POs have also been related to xylogenesis (Fukuda & Komamine, 1982; Miller *et al.*, 1985) as they are involved in the last step of lignin formation (Goldberg *et al.*, 1985; Imberty *et al.*, 1985). Thus, in addition to their possible role in the oxidation of IAA, the observed increase of PO activity during rooting might be due to the process of cell wall genesis inherent to cell division. This assumption is supported by the observation that the cell wall always indicated the presence of PO histologically.

The relative high level of PO activity in the other treatments could be caused by wounding, kinetin or some unknown causes. Gaspar *et al* (1997) presented the evidence that rooting and flowering were accompanied by similar biochemical changes, i.e. free IAA and putrescine levels, and peroxidase activity.

4.6.2 Polyphenol oxidase (PPO)

Injury, along with various stress factors, is known to trigger an increase in PPO activity (Mayer & Harel, 1979). This is probably one of the reasons why PPO activity increased in all the cases during the first four days in this study. PPO may oxidize endogenous or applied phenolics which then conjugate with oxidation products of auxin to form cofactors necessary for rooting (Bhattacharya, 1988). This is supported by the investigation that rooting could be improved by the oxidized forms of various phenolics (Poapst & Durkee, 1967). In the rooting system by cuttings, endogenous or applied phenolics are easily oxidized because cell damage by excision allows the enzyme and substrates to come into contact, thereby facilitating the oxidation of phenolics (Hand, 1994). However, PPO has been suggested to play a role in plant cell differentiation by its involvement in lignin biosynthesis (Haissig, 1986).

It may be noted that when the meristematic loci were formed at day 7, PPO activity was particularly pronounced. In fact, the cells that were involved in root primordium initiation were very limited at this stage. Therefore, high PPO activity in the cells that were involved in rooting could be diluted by others cells in conventional enzyme assays. This is supported by the accompanying histological investigation. The increase of PPO activity at the sites of root primordial initiation was obviously observed at day 6, and the strongest reaction was found at day 7 with increasing cell number. This is consistent with the enzyme activity determination by the colorimetric assay of tissue extracts. Unlike PO having strong reaction at cell walls, PPO exhibited high activity in cytoplasm. This is similar to PPO localization in pistachio (*Pistacia vera*) stem cuttings during adventitious root formation (Al Barazi & Schwabe, 1984). The presence of this enzyme at the actual sites of root primordial initiation might indicate a correlation between PPO and rooting. Therefore, although PPO seems to indirectly influence rooting through effects of rooting co-factors on cellular differentiation (Kaminski, 1971; Bassuk *et al.*, 1981; Haissig, 1986), it has potential as

a biochemical marker for root initiation in radiata pine, particularly at the histological level which permits early identification of root forming cells.

4.6.3 IAA-oxidase (IAA-O)

The result indicates that the increase of IAA-O activity was not only in response to rooting but also to other physiological process or wounding. This postulation is indirectly supported by the fact that IAA level declined before or during floral initiation (Bernier *et al.*, 1981; Pavlová & Krekule, 1984; Gaspar *et al.*, 1985b). Nevertheless, some investigations showed that IAA-O was closely related to root formation (Chibbar *et al.*, 1974 1979; Frenkel & Hess, 1974; Bansal & Nanda, 1981). It is reported that IAA could be oxidized by peroxidase in the presence of O_2, Mn^{2+} and a monophenol (Hinman & Lang, 1965; Endo, 1968; Pressey, 1990). Therefore, some POs have been considered to act as IAA-Os (Bhattacharya, 1988).

However, there have been reports of IAA-Os without PO activity (van der Mast, 1970; Shimokawa, 1983) and of PO failing to oxidize IAA (Lockhart, 1955; Huang & Haard, 1977). The current study indicates that PO might not be associated with IAA oxidization because PO activity was relatively high at day 0, while the same extracts assayed for IAA-O activity was very low at that time and the enzymatic patterns during the experimental period were different as well. Recently, Pan & Tian (1999) demonstrated that the activities of PO and IAA-O were positively correlated to rooting in mung bean hypocotyls as the activities of these two enzymes in the rooting zone coincided with root development. However, they suggested that the major role of IAA-O differed from that of PO in adventitious root formation.

4.6.4 Amylase (Amy)

As mentioned previously, starch may play an important role in the process of adventitious root formation. Amy is the enzyme responsible for the hydrolysis of starch into sugar, which is subsequently used as energy and carbon skeletons for root initiation and development. In the current study, the pattern of Amy activity in control was significantly different from that in the other treatments. However, the patterns in the treatments with IBA and IBA+kinetin were similar although their levels

were slightly different. Also, the Amy activity in the treatment with kinetin alone was similar to this pattern before day 7.

The result suggests that both IBA and kinetin may promote the fluctuation of amylase activity. This means that amylase is not directly related to root formation in the de-rooted radiata pine hypocotyls. Upadhyaya *et al* (1986) also gave the same assumption in cuttings of *Phaseolus vulgaris*. However, some reports indicate that there is a close correlation between the increased amylase activity and root formation (Nanda & Anand, 1970; Molnar & LaCroix, 1972b; Bhattacharya *et al.*, 1978).

4.6.5 Succinic dehydrogenase (SDH)

Few studies on the relationship between SDH and adventitious root formation have been reported. In *Hydrangea macrophylla*, SDH exhibited a high activity with the onset of root primordial initiation and the activity continued to increase with time (Molnar & LaCroix, 1972b). Avers (1958) also detected SDH activity in the meristematic cells of roots. Surprisingly, a report on SDH activity determination in cell-free extracts has not been found at least in the study of adventitious root formation.

An attempt was made to assay the SDH activity in the extracts of radiata pine hypocotyls. This is based on several studies of SDH in animal tissues. The principle is that SDH can catalyze triphenol-tetrazolium salt and produce red precipitate (reduced form), and ascorbic acid can also catalyze triphenol-tetrazolium salt to insoluble reduced tetrazolium. Therefore, the amount of reduced tetrazolium salt by SDH could be estimated with a standard curve, which is produced by a series of triphenol-tetrazolium chloride reduced by excess ascorbic acid. The results indicate that SDH activity increased in all the treatments, including growth regulator-free control, during the first day after excision of the hypocotyls. This seems reasonable because respiration of the cuttings may be enhanced by wounding and/or plant growth regulators. After this, the pattern of this enzyme activity in the growth regulator-free control is different from other treatments.

However, this is not consistent with the histochemical observations, in which SDH significantly increased in the meristematic tissue at day 5 in the treatment with

IBA. This may be due to the different limitations of the two methods. Thus the enzyme activity in cell-free extract represents the contributions from all cells of the hypocotyl tissue, but the enzyme activity in individual cells might not be high enough to show up histologically at day 1. Conversely, later on when the enzyme activity increased markedly in some specific cells such as meristematic cells but could have dropped in the majority of other cells, SDH activity determination in the cell-free extracts would indicate a low level of the enzyme activity as a result of the dilution effect of the conventional biochemical method of enzyme assay.

The histochemical location of this enzyme allows a close look at the changes of SDH activity in the cells involved in adventitious root formation. In the present study, the enzyme could be detected at the site where the root initiated at day 5, and seemed to increase with time. In *Hydrangea macrophylla*, the increase of this enzyme occurred before the first cell division, i.e. 2 to 3 days after the cuttings were made, and it started to increase noticeably in the region of root initiation (Molnar & LaCroix, 1972b).

4.7 Identification of genes expressed during rooting

The small amount of the proteins identified by 2D-PAGE led to use more powerful molecular techniques to clone and characterize the genes specifically expressed during adventitious root formation. To achieve this objective, the strategy of subtraction was employed.

4.7.1 PCR optimization

Initially the standard PCR conditions, including the reaction mix and the cycling parameters were those of Foote *et al.* (1997). However, only a very low molecular weight smear was present when 10 µl of the PCR product was analysed on an agarose gel. Therefore, optimization of PCR was attempted according to the suggestions by Guevara-Garcia *et al.* (1997). The effect of adding DMSO, DTT and Mg^{2+} to the reaction mix was evaluated. It seemed that the best yield and/or specificity of the PCR products were obtained to the highest when the concentration of Mg^{2+} was increased to 2.5 mM.

4.7.2 PCR product cloning

Approximately 30 white colonies were screened for the inserts, but insert longer than 700 bp was never observed. It is therefore possible that some of the PCR products with higher molecular weight were not cloned. There were two possible reasons why longer inserts were not obtained in this cloning system. Firstly, the longer cDNAs were more difficult to ligate to the vector than the shorter ones. Secondly, large recombinant (when long cDNAs were ligated to the vector) failed to enter the bacterial cell during the process of transformation. This could be overcome using λ phage cloning system or a small T-vector. For instance, Hutchison *et al.* (1999) prepared a T-vector from *Sma*I digest pUC19 DNA as described by Marchuk *et al.* (1990) and successfully cloned a near-full-length loblolly pine α-expansin cDNA (1046 bp).

4.7.3 IBA regulated gene expression during adventitious root formation

The main objective of this investigation was to identify, via their cDNAs, genes that are preferentially activated upon the induction of adventitious root formation, and could play a crucial role in the initiation of this process. As expected, a number of cDNAs was identified which are likely to correspond to a group of genes associated with adventitious root formation.

The pattern of Southern hybridization of radiata pine DNA with the probe R2-7 indicated that the corresponding gene might be represented by a single gene or a member in a small gene family. Deducing from the hybridization pattern, it seems that one or two *Eco*RI sites are present in the sequence of this gene. However, this could be negated by the fact that the probe was labelled by PCR using *Eco*RI digested plasmid DNA as template and a single oligo d(T) with stablizer as primer (*Eco*RI PCR primer). If any *Eco*RI site exists in this sequence, the PCR cannot be successfully performed after *Eco*RI digestion. The additional bands found in *Eco*RI lane may have been due to sites in introns of this gene or due to cross hybridization with closely related genes. Similar results were also obtained by other researchers (Kelly *et al.*, 1990; Chen *et al.*, 1996; Butler & Gallagher, 1999). For example, two *Eco*RI bands

were detected when digested mungbean genomic DNA was probed with 2 cDNAs although there is no *Eco*RI site in these two cDNAs (Chen *et al.*, 1996).

The probes here were chosen randomly. Most likely, additional clones corresponding to genes expressed in earlier or later stages can be found following cDNA library screening with the subtracted ss-cDNAs or the derived PCR products.

Research on gene expression during adventitious root formation in radiata pine has just been started as described in this study. Further characterization of the genes associated with the process and analysis of their possible functions could contribute to the understanding of the molecular mechanisms underlying the complex process of adventitious root formation, not only in radiata pine but also in other plants.

4.7.4 Identification of the cloned genes associated with rooting in other species

Chen *et al.* (1996) isolated two different cDNAs (*MII-3* and *MII-4*) from a cDNA library made from mungbean hypocotyls treated with IAA. Expression of these two genes could be stimulated with increasing concentrations of IAA from 100 to 1000 μM, but was not observed in rooting control and IAA-treated leaves. The authors compared the nucleotide and predicted amino acid sequences with other reported genes using the BLAST programs (Altschul *et al.*, 1990). There were no significant similarities between *MII-3* and other genes or proteins in the database. However, the similarities between the deduced amino acid sequence of *MII-4* and a heat shock protein from soybean (M20363) (Czarnecka *et al.*, 1988), an auxin-induced protein from soybean (J03197) (Hagen *et al.*, 1988) and an auxin-induced protein from tobacco (X56264) (van der Zaal *et al.*, 1991) were 80%, 79% and 52%, respectively.

Hutchison *et al.* (1999) identified a gene family whose mRNA levels increased in hypocotyl cuttings of loblolly pine in response to the application of IBA. A BLAST search (Altschul *et al.*, 1990) of the database revealed this gene family is the loblolly pine homolog of the α-expansins, which have previously been found in the monocots and dicots (McQueen-Mason *et al.*, 1992; Shcherban *et al.*, 1995). They are thought

to be responsible for acid-induced loosening of cellulose-hemicellulose networks (McQueen-Mason *et al.*, 1992; Rayle & Cleland, 1992; Cosgrove & Li, 1993; McQueen-Mason, 1995). In contrast to the previous reported expansin expression in rapid growing areas of the plants, α-expansin-related sequences were induced in non-growing regions of loblolly pine hypocotyls prior to the resumption of cell division leading to adventitious root formation.

In apple stem discs, Butler & Gallagher (1999) characterized a cDNA fragment which was differentially expressed during adventitious root formation. Sequence analysis of this cDNA clone indicates that it encodes a 2-oxoacid-dependent dioxygenase (2-ODD), designated *ARRO-1* (Adventitious Rooting Related Oxygenase). *ARRO-1* is up-regulated during the previously identified induction phase (24-96 hours) of adventitious root formation (De Klerk *et al.*, 1995), suggesting that it may function as a component of auxin-triggered, rooting-specific gene cascades (Butler & Gallagher, 1999).

Carpin *et al.* (1999) isolated and cloned a cDNA, encoding a calcium-pectate-binding anionic isoperoxidase (APRX), from etiolated zucchini hypocotyls. APRX protein and mRNA were present in root, hypocotyls and cotyledons. *In situ* hybridization showed that the APRX-encoding gene was expressed in many different tissues, including lateral and adventitious root primordia. Based on this observation, the author suggested that APRX could be involved in lignin formation and the transcription of its gene was related to auxin level.

Genes that are specific for the initial phases of rooting were also identified in lateral root primordia (Taylor & Scheuring, 1994; Vera *et al.*, 1994; Smith & Fedoroff, 1995). For instance, a gene, designated *LRP1* for lateral root primordium 1, encodes a novel protein and its expression is activated during the early stages of lateral root primordium development and adventitious root development as well (Smith & Fedoroff, 1995). Based on several observations, the author suggested that *LRP1* is a member of a small gene family with overlapping or redundant functions and its expression may be regulated by auxin during early lateral root primordium development.

So far, different rooting-related cDNAs have been isolated and characterized in several plant species. Therefore, the current information suggests that multi-genes might be involved in the process of adventitious rooting, or different genes might be activated in response to adventitious root formation in different plant species. Overall, information about the expression of the rooting related-genes is still limited. Further comprehensive characterization of these genes is required for better understanding of adventitious root formation at the molecular level.

4.8 Root induction by *Agrobacterium rhizogenes*

This study showed that *A. rhizogenes* seemed to improve the root formation of *P. radiata*. Although strain A4T was effective in increasing rooting in some treatments, the overall observation was that strain LBA9402 was more effective in increasing both rooting percentage and root number. McAfee *et al.* (1993) and Hatta *et al.* (1996) also found that different strains of *A. rhizogenes* gave different responses in pine (*Pinus*) spp. and larch (*Larix*) spp., and in jujube (*Ziziphus jujuba*).

Apart from the differences due to different strains, the results here also show that different methods of inoculation with the bacterium significantly affected survival percentages and the consequent rooting percentages and root number.

Interestingly, the results of M1 indicated that supplement of IBA in the culture medium significantly increased rooting percentage and root number. This suggests that in this system the endogenous auxin level is not high enough for root formation, although *A. rhizogenes* carries genes that encode auxin synthesis or increase auxin sensitivity to plant tissue. In contrast, the endogenous auxin level is relatively high for root formation in M2 after inoculation with the bacteria because plant materials used were intact seedlings.

In addition, M1 may be a good system for gene transformation as a small amount of tissue is required and the progress of root formation is easily observed. Unfortunately, when the roots formed in this treatment were excised, they could not survive on hormone-free medium in several attempts in this study. β-Glucuronidase (GUS) activity was also not detected in the roots induced by LBA9402 which carries

this reporter gene. Surprisingly, roots induced by LBA9402 on tobacco leaf discs, as a control, indicated negative β-Glucuronidase (GUS) activity as well (data not shown). Therefore, there may exist two possibilities in response to the results. First, the GUS gene was lost in LBA9402 during transportation or culturing. Second, the induced roots were not genetically transformed. McAfee *et al.* (1993) did not detect the T-DNA in the induced roots of *Pinus monticola*, *P. banksiana* and *Larix laricina* using the left T-DNA region of pRiA4b as the DNA probe. The authors suggested that no T-DNA transfer had occurred or that it was present at a level lower than that detectable by the Southern blot procedure. Bassil *et al.* (1991) were unable to determine if the roots induced on hazelnut cuttings contained transformed tissue after opine and molecular analysis for TR- and TL-DNA. In the present study, it seems that the induced roots were not genetically transformed, but root induction was probably due to an improvement of rooting environment by the bacterium as suggested by other workers (Simpson *et al.*, 1986; McAfee *et al.*, 1993). Recently, similar results have been obtained by Falasca *et al.* (2000) who conducted an experiment to investigate how *A. rhizogenes* triggers *de novo* root formation in a recalcitrant woody walnut plant (*Fuglans regia*). They found that rooting on the infected cuttings was enhanced by application of exogenous IBA, which accelerated and increased root meristemoid formation, in comparison with the growth regulator-free treatment. PCR and Southern blotting analysis showed that root meristemoid formation was not due to transformation. Thus, in a recalcitrant walnut, non-transformed root meristemoids are stimulated by the combination of the bacterium infection and exogenous application of IBA.

Strain LBA9402 significantly increased rooting percentage and root number in adventitious shoots. This application, combined with other treatments, may be useful for vegetative propagation of radiata pine by rooting adventitious shoots.

Chapter 5

General discussion and conclusions

Like in many other plant species, application of exogenous auxin is required to achieve adventitious rooting in cuttings of *Pinus radiata*. IBA may enter the cuttings predominantly via the cut surface and reach the 'target cells', which are located at the margin of a differentiating resin duct or parenchyma tissue external to this tissue. When the competent cells are stimulated by rooting signal such as IBA, a series of biochemical and molecular events are set in motion. These could start before the first cell division although no significant histological changes were observed at this stage. For example, the expressions of some genes might be turned on in these cells and their gene products could prepare cell for the first division. But, due to the limited number of cells involved most biochemical changes could not be detected at the early stage, even using histological localization. *In situ* hybridization may provide a valuable tool for detection of individual cells expressing a few mRNA molecules per cell (Angerer *et al.*, 1987).

In *P. radiata*, genes encoding PO, PPO, Amy, SDH and starch synthesis related enzymes may be activated or enhanced at the same time or differentially. The content of total buffer soluble proteins (including enzymes) in the rooting treatment started to increase at day 1 and reach its maximum at 7. Compared with a rooting control, 19 proteins were identified to be associated with the IBA treatment or root formation using 2D-PAGE, although these proteins were of such small magnitudes so that it was difficult to quantify them and establish them as novel proteins. This does not diminish the possible importance of some of these changes. In fact, small changes in protein synthesis, for example enzymes, may cause large changes in developmental systems.

Carbohydrates are important for adventitious root formation in *P. radiata*. In the treatment with IBA (rooting treatment), a significant increase of starch was observed both in tissue extract and at the histological level. Histochemical localization of starch, particularly, indicated the importance of starch to root initiation and development, as the starch grains were closely associated with root primordium initiation. Therefore, it is possible to improve rooting of radiata pine cuttings by exogenously application of carbonhydrates, such as sucrose.

The hybridization experiments with the probes R2-7, R2-22 and R2-35 indicated that the corresponding genes are expressed early on IBA-induced adventitious root formation in radiata pine hypocotyls. It is possible that the expression of R2-7 and R2-35 begin earlier than day 4, for instance at day 2 or day 3 if these samples were analysed.

Agrobacterium rhizogenes may significantly induce adventitious root formation in *P. radiata* hypocotyls and shoots though the mechanism responsible for this effect is still not known in the present study.

Based on the data obtained in this study, the following conclusions may be made.

1. Unlike most other plant species which do not require exogenous auxin after the induction phase, the cuttings of *P. radiata* require continuous application of exogenous auxin (IBA) to further support the growth of meristematic tissues leading to root primordia. For high root counts in this rooting system, hypocotyl cuttings may be treated with 6-9 mg/l of IBA for 10 days.

2. After the rooting treatment (IBA), competent cells begin to divide around day 4. Root primordial initials and well-organized primordia are formed at day 7 and 10, respectively. The root primordia grow through cortex and become visible by around day 13.

3. Due to the small magnitude of protein changes, proteins that are related to root formation could not be detected by 1D SDS-PAGE. But about 19

proteins that were associated with IBA treatment were identified by the more powerful 2D-PAGE technique.

4. Carbohydrates are very important to adventitious root formation. Starch accumulation can be enhanced by exogenous application of sucrose. Significantly more starch accumulated in the rooting treatment than the non-rooting controls from day 4 to day 7. The starch accumulation was related to the area of root initiation and could be a marker of adventitious root formation in *P. radiata.*

5. PO, PPO, Amy and SDH might be involved in the process of adventitious root formation. PO, PPO and SDH may be potential biochemical rooting markers at the histological level.

6. The small-scale study on gene expression indicated that the transcripts represented by three cloned cDNAs (i.e. R2-7, R2-22 and R2-35) seem to be related to adventitious root formation. The expression of the genes represented by the cDNA clones are transient under rooting conditions, i.e. turning on and off at certain times. Southern blotting analysis indicated that the R2-7 transcript isolated in this study is likely represented by a single gene or from a small gene family.

7. Adventitious root formation of both hypocotyl tissue and adventitious shoots can be improved significantly by inoculation with *A. rhizogenes.*

However, more work is required in order to further understand root induction by *A. rhizogenes* and fully characterize the regulation of gene expression during adventitious root formation.

More work should be directed to obtain full length of cDNAs associated with adventitious root formation. In particular, it is desirable to find the cDNAs representing the two extra RNA bands that were present at day 4 and 7 on the agarose gel. Furthermore, a cDNA library can be made from hypocotyls treated with IBA for less than 7 days (e.g. 4 or 5 days) to look for genes whose expression might be enhanced prior to root primordium formation.

Current data suggest that R2-7 and R2-35 may prove to be a reliable molecular marker for adventitious root formation of *Pinus radiata*. With this purpose in mind, the expression patterns of these two genes should be characterized in depth.

The effects of different rooting treatments (different concentrations of IBA resulting in various root counts, anti-auxin: TIBA, PCIB, PBA or POAA, inoculation of *Agrobacterium rhizogenes*) on the gene expression should be further investigated. For example, the treatment with TIBA drastically lowered the level of a transcript (mRNA encoding anionic isoperoxidase – APRX) that could be extracted from the rooting region of hypocotyls in rooted zucchini seedlings (Carpin *et al.*, 1999). In mungbean hypocotyls, the IAA dose-dependent rooting response coincided with increases in expression of *MII*-3 and *MII-4*, two auxin-regulated genes isolated from mungbean hypocotyls during adventitious root formation (Chen *et al.*, 1996).

In situ hybridization has become sufficiently sensitive to detect mRNAs present at the level of only a few molecules per cell, allowing the identification of individual cells expressing specific genes (Angerer *et al.*, 1987). Thus, the expression of rooting specific genes can be investigated at the histological level in different tissues, including hypocotyls, epicotyls, roots and adventitious shoots, etc. In particular, attention should be paid to those cells that initiate the formation of root primordia at early stage.

After screening the cDNA library and Northern blotting analysis, several potential cDNAs may be sequenced. The complete nucleotide and deduced amino acid sequence can be compared with other reported genes by accessing related database. If the comparisons show significant similarity to other genes or proteins, the properties of the genes may be deduced. Otherwise, further characterization of the genes is needed. In this way, the roles of these genes in rooting process may be better understood.

Molecular cloning and characterization of the genes expressed during adventitious root formation of radiata pine are important for understanding the molecular mechanism of this process. Furthermore, the studies of gene expression

will reveal the reason why why some clonal cuttings of radiata pine root more easily than others do, and why mature cuttings are difficult for adventitious root induction.

It has been shown here that adventitious root formation in both hypocotyls and adventitious shoots of *Pinus radiata* could be enhanced by inoculation of *A. rhizogenes*. As a practical method, further research is required to seek the appropriate approach to improve the vegetative propagation of this species and the quality of the subsequent root system. For instance, other rooting treatments (Horgan & Aitken, 1981; Aitken-Christie *et al.*, 1985; Horgan & Holland, 1989) in combination with *A. rhizogenes* inoculation may be worth considering.

It is unclear whether the increased root formation in this study was due to gene transfer, although β-glucuronidase (GUS) activity was not detected in the roots induced by LBA9402. Furthermore, GUS activity was also not detected in LBA9402 induced roots on tobacco leaf discs (data not shown). Perhaps, the strain used was deficient in the GUS gene construct. To examine the possibility that gene transfer occurred in the treatment with *A. rhizogenes*, Southern blotting and PCR analysis are necessary. DNAs may be extracted from induced roots and normal roots as a control. After digestion with restriction endonucleases, the DNAs may be separated on agarose gel, blotted to a nylon membrane and hybridized with an *Eco*RI fragment of 4.3 kb from pRi T-DNA including *rolA, B* and *C* (open reading frames 10, 11 and 12) (Nin *et al.*, 1997). PCR may also be performed with either NTP II-gene-specific primers (Nin *et al.*, 1997) or GUS-gene-specific primers (Tzfira *et al.*, 1996a) in the case of LBA9402. Direct Southern hybridization without PCR amplification may not detect low level of transformed cells (Strobel *et al.*, 1988), but after PCR amplification hybridization signal may be detected if some cells are transformed.

In conclusion, information obtained from the studies on transformation or root induction by *A. rhizogenes* will not only improve the adventitious root formation ability of a 'difficult-to-root' species, but alternatively also provide a model to study the molecular control of adventitious root formation in woody plants.

References

Ackermann, C. (1977). Pflanzen aus *Agrobacterium rhizogenes* tumoren aus *Nicotiana tabacum*. *Plant Sci. Lett.* 8:23-30.

Aitken-Christie, J., Jones, C. & Bond, S. (1985). Wet and waxy shoots in radiata pine micropropagation. *Acta Hortic.* 166:93-100.

Al Barazi, Z. & Schwabe, W. W. (1984). The possible involvement of polyphenol-oxidase and the auxin-oxidase system in root formation and development in cuttings of *Pistacia vera*. *J. Hort. Sci.* 59:453-461.

Aleith, F. & Richter, G. (1990). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. *Planta* 183:17-24.

Allsop, F. (1950). Propagation of *Pinus radiata* by means of cuttings. *N. Z. For. Serv. Res. Notes* 1.

Altman, A. & Bachrach, U. (1981). Involvement of polyamines in plant growth and senescence. In Caldarera, C. M., Zappia, V. & Bachrach, U., eds. *Advances in Polyamine Research.* pp 365-375. Raven Press. New York, USA.

Altman, A. & Wareing, P. F. (1975). The effect of IAA on sugar accumulation and basipetal transport of ^{14}C-labelled assimilates in relation to root formation in *Phaseolus vulgaris* cuttings. *Physiol. Plant.* 33:32-38.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.

Alvarez, R., Nissen, S. J. & Sutter, E. G. (1989). Relationship between indole-3-acetic acid levels in apple (*Malus pumila* Mill) rootstocks cultured *in vitro* and adventitious root formation in the presence of indole-3-butyric acid. *Plant Physiol.* 89:439-443.

Andersen, A. S. (1986). Environmental influences on adventitious rooting in cuttings of non-woody species. In Jackson, M. B., ed. *New Root Formation in Plants and Cuttings.* pp 223-253. Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster.

Angerer, L. M., Cox, K. H. & Angerer, R. C. (1987). Demonstration of tissue-specific gene expression by *in situ* hybridization. In Colowick, S. P. & Kaplan, N. O., eds. *Methods in Enzymology.* pp 649-661. Academic Press. New York.

Appelgren, M. (1976). Regeneration of *Begonia* × *Hiemalis in vitro*. *Acta Hortic.* 64:31-38.

Appelgren, M. & Heide, O. M. (1972). Regeneration in *streptocarpus* leaf discs and its regulation by temperature and growth substances. *Physiol. Plant.* 27:417-423.

Argles, G. K. (1959). Root formation and root development in stem cuttings: a re-examination of certain fundamental aspects. *Ann. Appl. Biol.* 47:626-628.

Audus, L. J. (1953). *Plant Growth Substances*. Leonard Hill. London.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989). *Current Protocols in Molecular Biology*. Vol. 1. Greene Publishing Associates and Wiley-Interscience. New York, NY.

Avers, C. J. (1958). Histochemical localization of enzyme activity in the root epidermis of *Phleum pratense*. *Amer. J. Bot.* 45:609-613.

Avidan, B. & Lavee, S. (1978). Physiological aspects of the rooting ability of olive cultivars. *Acta Hortic.* 79:93-101.

Bachelard, E. P. & Stowe, B. B. (1963). Rooting of cuttings of *Acer rubrum* L. and *Eucalyptus camaldulensis* Dehn. *Aust. J. Bio. Sci.* 16:751-767.

Baleriola-Lucas, C. & Mullins, M. (1984). Micropropagation of two Frech prune cultivars. *Agronomie* 4:473-477.

Bansal, M. P. & Nanda, K. K. (1981). IAA oxidase activity in relation to adventitious root formation on stem cuttings of some forest tree species. *Experientia* 37:1273-1274.

Baraldi, R., Bertazza, G., Bregoli, A. M., Fasolo, F., Rotondi, A., Predieri, S. & Serafini-Fracassini, D. (1995). Auxins and polyamines in relation to differential *in vitro* root induction on microcuttings of two pear cultivars. *J. Plant Growth Regul.* 14:49-59.

Barlow, P. W. (1986). Adventitious roots of whole plants: their forms, functions, and evolution. In Jackson, M. B., ed. *New Root Formation in Plants and Cuttings.* pp 67-110. Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster.

Barlow, P. W. (1994). The origin, diversity and biology of shoot-borne roots. In Davis, T. D. & Haissig, B. E., eds. *Biology of Adventitious Root Formation.* pp 1-23. Plenum Press. New York.

Bassil, N. V., Proebsting, W. M., Moore, L. W. & Lightfoot, D. A. (1991). Propagation of hazelnut stem cuttings using *Agrobacterium rhizogenes*. *HortScience* 26:1058-1060.

Bassuk, N. L. & Howard, B. H. (1981). A positive correlation between endogenous root-inducing co-factor activity in vacuum extracted sap and seasonal changes in M26 winter apple cuttings. *J. Hort. Sci.* 56:301-312.

Bassuk, N. L., Hunter, L. D. & Howard, B. H. (1981). The apparent involvement of polyphenol oxidase and phloridzin in the production of apple rooting cofactors. *J. Hort. Sci.* 56:313-322.

Basu, R. N., Bose, T. K., Roy, B. N. & Mukhopadhyay, A. (1969). Auxin synergists in rooting of cuttings. *Physiol. Plant.* 22:649-652.

Batten, D. J. & Goodwin, P. B. (1978). Phytohormones and the induction of adventitious roots. In Letham, D. S., Goodwin, P. B. & Higgins, T. J. V., eds. *Phytohormones and Related Compound --A Comprehensive Treaties.* pp 137-173. Elsevier/North-Holland Biomedical Press. Amsterdam.

Batten, D. J. & Mullins, M. G. (1978). Ethylene and adventitious root formation in hypocotyl segments of etiolated mung-bean (*Vigna radiata* (L.) Wilcxek) seedlings. *Planta* 138:193-197.

Bellamine, J., Penel, C., Greppin, H. & Gaspar, T. (1998). Confirmation of the role of auxin and calcium in the late phases of adventitious root formation. *Plant Growth Regul.* 26:191-194.

Ben-Efraim, I., Gad, A. E., Cohen, P., Reymond, P. & Pilet, P. E. (1990). The effect of 4-chlororesorcinol on the endogenous levels of IAA, IBA and oxidative enzymes in cuttings. *Plant Growth Regul.* 9:97-106.

Bernfeld, P. (1955). Amylases, α and β. In Colowick, S. P. & Kaplan, N. O., eds. *Methods in Enzymology.* pp 149-158. Academic Press. New York.

Bernier, G., Kinet, J.-M. & M, S. R. (1981). *The physiology of flowering.* Vol. II. CRC Press. Boca Raton.

Berthon, J. Y., Maldiney, R., Sotta, B., Gaspar, T. & Boyer, N. (1989). Endogenous levels of plant hormones during the course of adventitious rooting in cuttings of *Sequoiadendron giganteum* (Lindl) in vitro. *Biochem. Physiol. Pflanzen* 184:405-412.

Bhattacharya, N. C. (1988). Enzyme activities during adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 88-101. Dioscorides Press. Portland, Oregon.

Bhattacharya, N. C., Bhattacharya, S. & Malik, C. P. (1976a). Transients in the activities of some hydrolases and their relationship with adventitious root formation in hypocotyl cuttings of *Phaseolus mungo. Plant Sci.* 8:38-44.

Bhattacharya, N. C., Bhattacharya, S. & Nanda, K. K. (1978). Isoenzyme polymorphism of peroxidase, IAA-oxidase, catalase and amylase in rooting etiolated stem segments of *Salix tetrasperma. Biochem. Physiol. Pflanzen* 172:439-452.

Bhattacharya, N. C., Parmar, S. S. & Nanda, K. K. (1976b). Isoenzyme polymorphism of amylase and catalase in relation to rooting etiolated stem segments of *Populus nigra. Biochem. Physiol. Pflanzen* 170:133-142.

Bhattacharya, N. C., Parmar, U., Malik, C. P. & Bhattacharya, S. (1977). Paradoxical effects of actinomycin-D in rooting hypocotyl cuttings of *Phaseolus mungo. Z. Pflanzenphysiol.* 85:377-382.

Bhattacharya, S. & Nanda, K. K. (1978). Stimulatory effect of purine and pyrimidine bases and their role in the mediation of auxin action through the regulation of carbohydrate metabolism during adventitious root formation in hypocotyl cuttings of *Phaseolus mungo*. *Z. Pflanzenphysiol.* 88:283-293.

Bhattacharya, S. & Nanda, K. K. (1979). Promotive effect of purine and pyrimidine bases and their role in the mediation of auxin action through regulation of oxidases and phosphatases in rooting cuttings of *Phaseolus mungo*. *Indian J. Exp. Biol.* 17:40-45.

Bhella, H. S. & Roberts, A. N. (1975). Seasonal changes in origin and rate of development of root initials in Douglas-fir stem cuttings. *J. Amer. Soc. Hort. Sci.* 100:643-646.

Biondi, S., Diaz, T., Iglesias, I., Gamberini, G. & Bagni, N. (1990). Polyamines and ethylene in relation to adventitious root formation in *Prunus avium* shoot cultures. *Physiol. Plant.* 78:474-483.

Biran, I. & Halevy, A. H. (1973a). Endogenous levels of growth regulators and their relationship to the rooting of dahlia cuttings. *Physiol. Plant.* 28:436-442.

Biran, I. & Halevy, A. H. (1973b). The relationship between rooting of dahlia cuttings and the presence and type of bud. *Physiol. Plant.* 28:244-247.

Biran, I. & Halevy, A. H. (1973c). Stock plant shading and rooting of *Dahlia* cuttings. *Scientia Hortic.* 1:125-131.

Bláhová, M. (1969). Changes in the level of endogenous gibberellins and auxin preceding the formation of adventitious roots on isolated epicotyls of pea plants. *Flora* 160:493-499.

Blake, T. J. & Atkinson, S. M. (1986). The physiological role of abscisic acid in the rooting of poplar and aspen stump sprouts. *Physiol. Plant.* 67:638-643.

Blakesley, D., Weston, G. D. & Hall, J. F. (1991). The role of endogenous auxin in root initiation. I. Evidence from studies on auxin application and analysis of endogenous levels. *Plant Growth Regul.* 10:341-353.

Blazich, F. A. (1988). Mineral nutrition and adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 61-69. Dioscorides Press. Porland, Oregon, USA.

Blazich, F. A. & Wright, R. D. (1979). Non-mobilization of nutrients during rooting of *Ilex crenata* Thunb. cv. Convexa stem cuttings. *HortScience* 14:242.

Blazich, F. A., Wright, R. D. & Schaffer, H. E. (1983). Mineral nutrient status of 'Convexa' holly cuttings during intermittent mist propagation as influenced by exogenous auxin application. *J. Amer. Soc. Hort. Sci.* 108:425-429.

Bollmark, M. & Eliasson, L. (1986). Effects of exogenous cytokinins on root formation in pea cuttings. *Physiol. Plant.* 68:662-666.

Borthwick, H. A., Hamner, K. C. & Parker, M. W. (1937). Histological and microchemical studies of the relation of tomato plants to indole-3-acetic acid. *Bot. Gaz.* 98:491-519.

Böttger, M. (1978). Levels of endogenous indole-3-acetic acid and abscisic acid during the course of the formation of lateral roots. *Z. Pflanzenphysiol.* 86:283-286.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Breen, P. J. & Muroaka, T. (1973). Effect of indolebutyric acid on distribution of ^{14}C-photosynthate in softwood cuttings of 'Mariana 2624' plum. *J. Amer. Soc. Hort. Sci.* 98:436-439.

Breen, P. J. & Muroaka, T. (1974). Effect of leaves on carbohydrate content and movement of ^{14}C-assimilates in plum cuttings. *J. Amer. Soc. Hort. Sci.* 99:326-332.

Brenner, C. A., Tam, A. W., Nelsson, P. A., Engleman, E. G., Suzuki, N., Fry, K. E. & Larick, J. W. (1989). Message amplification phenotyping (MAPPing): A technique to simultaneously measure multiple mRNAs from small numbers of cells. *BioTech.* 7:1096-1103.

Bridglall, S. S. & van Staden, J. (1984). Effect of auxin on rooting and endogenous cytokinin levels in leaf cuttings of *Phaseolus vulgaris* L. *J. Plant Physiol.* 117:287-292.

Brunner, H. (1978). Influence of various growth substances and metabolic inhibitors on root regenerating tissue of *Phaseolus vulgaris* L. Changes in the contents of growth substances and in peroxidase and IAA oxidase activities. *Z. Pflanzenphysiol.* 88:13-23.

Brutsch, M. O., Allan, P. & Wolstenholme, B. N. (1977). The anatomy of adventitious root formation in adult-phase pecan (*Carya illinoensis* (Wang) K. Koch) stem cuttings. *Hort. Res.* 17:23-31.

Burritt, D. J. (1992). Studies on organogenesis from explants of *Begonia erythrophylla*. *Ph.D Thesis.* Department of Plant & Microbial Sciences, University of Canterbury, Christchurch.

Butler, E. D. & Gallagher, T. F. (1999). Isolation and characterization of a cDNA encoding a novel 2-oxoacid-dependent dioxygenase which is up-regulated during adventitious root formation in apple (*Malus domestica* 'Jork 9') stem discs. *J. Exp. Bot.* 50:551-552.

Cameron, R. J. (1968). The propagation of *Pinus radiata* by cuttings: influences affecting the rooting of cuttings. *N.Z. J. For.* 13:78-89.

Cameron, R. J. & Thomson, G. V. (1969). The vegetative propagation of *Pinus radiata*: root initiation in cuttings. *Bot. Gaz.* 130:242-251.

Cano, A., Artés, F., Arnao, M. B., Sánchez-Bravo, J. & Acosta, M. (1996). Inhibition of etiolated lupin hypocotyl growth and rooting by peroxidase, ascorbate and glutathione. *J. Plant Physiol.* 147:721-728.

Capone, I., Spanò, L., Cardarelli, M., Bellincampi, D., Petit, A. & Costantino, P. (1989). Induction and growth properties of carrot roots with different complements of *Agrobacterium rhizogenes* T-DNA. *Plant Mol. Biol.* 13:43-52.

Carlson, M. C. (1929). Origins of adventitious roots in coleus cuttings. *Bot. Gaz.* 87:119-126.

Carpenter, J. B. (1961). Occurrence and inheritance of preformed root primordia in stems of citron (*Citrus medica* L.). *Proc. Amer. Soc. Hort. Sci.* 77:211-218.

Carpin, S., Crevecoeur, M., Greppin, H. & Penel, C. (1999). Molecular cloning and tissue-specific expression of an anionic peroxidase in zucchini. *Plant Physiol.* 120:799-810.

Chandra, G. R., Gregory, L. E. & Worley, J. F. (1971). Studies on the initiation of roots on mung bean hypocotyl. *Plant Cell Physiol.* 12:317-324.

Chandra, G. R., Worley, J. F., Gregory, L. E. & Clark, H. D. (1973). Effect of 6-benzyladenine on the initiation of adventitious roots on mung bean hypocotyl. *Plant Cell Physiol.* 14:1209-1212.

Chen, J., Wu, D., Witham, F. H., Heuser, C. W. & Arteca, R. N. (1996). Molecular cloning and characterization of auxin-regulated genes from mungbean hypocotyls during adventitious root formation. *J. Amer. Soc. Hort. Sci.* 121:393-398.

Chibbar, R. N., Gurumurti, K. & Nanda, K. K. (1974). Non-enzymic proteinaceous inhibitor of IAA oxidase in stem cutting of *Ipomoea fistulosa*. *Biochem. Physiol. Pflanzen* 165:325-330.

Chibbar, R. N., Gurumurti, K. & Nanda, K. K. (1979). Changes in IAA oxidase activity in rooting hypocotyl cuttings of *Phaseolus mungo* L. *Experientia* 35:202-203.

Chilton, M. D., Tepfer, D. A., Petit, A., Casse-Delbart, F. & Tempé, J. (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of host plant root cells. *Nature (London)* 295:432-434.

Chin, T., Meyer, M. M., Jr & Beevers, L. (1969). Abscisic-acid-stimulated rooting of stem cuttings. *Planta* 88:192-196.

Choi, Y. E. & Soh, W. Y. (1997). Effect of ammonium ion on morphogenesis from cultured cotyledon explants of *Panax ginseng*. *J. Plant Biol.* 40:21-26.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Christensen, M. V., Eriksen, E. N. & Andersen, A. S. (1980). Interaction of stock plant irradiance and auxin in the propagation of apple rootstock plants by cuttings. *Scientia Hortic.* 12:11-17.

Clegg, K. M. (1956). The application of the anthrone reagent to the estimation of starch in cereals. *J. Sci. Food Agric.* 7:40-44.

Coleman, W. K. & Greyson, R. I. (1977). Analysis of root formation in leaf discs of *Lycopersicon esculentum* Mill. cultured *in vitro*. *Ann. Bot.* 41:307-320.

Collet, G. F. & Le, C. L. (1987). Role of auxin during *in vitro* rhizogenesis of rose and apple-trees. *Acta Hortic.* 212:273-280.

Cosgrove, D. J. & Li, Z. C. (1993). Role of expansin in cell enlargement of oat coleotiles: Analysis of developmental gradients and photo-control. *Plant Physiol.* 103:1321-1328.

Crouch, M. L. & Bopp, M. (1984). Non-zygotic embryos of *Brassica napus* L. contain embryo specific storage proteins. *Planta* 156:520-524.

Curir, P., van Sumere, C. F., Termini, A., Barthe, P., Marchesini, A. & Dolci, M. (1990). Flavonoid accumulation is correlated with adventitious root formation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. *Physiol. Plant.* 92:1148.

Curry, E. A. & Williams, M. W. (1983). Promalin or GA_3 increase pesdicel and fruit length and leaf size of "Delicious" apples treated with paclobutrazol. *HortScience* 18:214-215.

Czarnecka, E., Nagao, R. T., Key, J. L. & Gurley, W. B. (1988). Characterization of *Gmhsp26,* a stress gene encoding a divergent heat shock protein of soybean: Heavy-metal-induced inhibition of intro processing. *Mol. Cell Bio.* 8:1113-1122.

Dalgas, K. F. (1973). Anatomical studies on cuttings of Norway spruce (*Picea abies* (L.) Karst.) undergoing the rooting process. *For. Tree Improv.* 5:1-20.

Davis, T. D. (1986). Influence of triazole growth retardants on adventitious root formation in bean hypocotyl cuttings. *Pro. Plant Growth Reg. Soc. Amer.* 13:217-223.

Davis, T. D. (1988a). Effect of shoot growth retardants and inhibitors on adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 174-184. Dioscorides Press. Portland, Oregon.

Davis, T. D. (1988b). Photosynthesis during adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 79-87. Dioscorides Press. Portland, Oregon.

Davis, T. D. & Potter, J. R. (1981). Current photosynthate as a limiting factor in adventitious root formation on leafy pea cuttings. *J. Amer. Soc. Hort. Sci.* 106:278-282.

Davis, T. D., Sankhla, N., Walser, R. H. & Upadhyaya, A. (1985). Promotion of adventitious root formation on cuttings by paclobutrazol. *Ibid.* 20:883-884.

De Cleene, M. & De Ley, J. (1981). The host range of infectious hairy root. *Bot. Rev.* 47:147-193.

De Jong, D. W. (1967). An investigation of the role of plant peroxidase in cell wall development by the histochemical method. *J. Histochem. Cytochem.* 15:335-346.

De Klerk, G., Ter Brugge, J., Smulders, R. & Benschop, M. (1990). Basic peroxidases and rooting in microcuttings of *Malus*. *Acta Hortic.* 280:29-36.

De Klerk, G. J. (1996). Markers of adventitious root formation. *Agronomie* 16:609-616.

De Klerk, G. J., van der Krieken, W. & De Jong, J. C. (1999). The formation of adventitious roots: New concepts, new possibilities (Review). *In Vitro Cell. Dev. Bio. Plant* 35:189-199.

De Klerk, G.-J., Keppel, M., Ter Brugge, J. & Meekes, H. (1995). Timing of the phases in adventitious root formation in apple microcuttings. *J. Exp. Bot.* 46:965-972.

Defendi, V. & Pearson, B. (1955). Quantitative estimation of succinic dehydrogenase activity in a single microscopic tissue section. *J. Histochem. Cytochem.* 3:61-70.

DeJong, T. M. & Doyle, J. F. (1984). Leaf gas exchange and growth responses of mature 'Fantasia" nectarine trees to paclobutrazol. *J. Amer. Soc. Hort. Sci.* 109:878-882.

Delegher-Langohr, V. (1974). Influence of five growth substances on the root formation by cuttings of *Zebrina pendula*. *Bull. Soc. Royal Bot. Belg.* 107:73-89.

Dhaliwal, G., Bhattacharya, N. C. & Nanda, K. K. (1974). Promotion of rooting by cycloheximide on hypocotyl cuttings of *Impatiens balsamina* and associated changes in the pattern of isoperoxidases. *Ind. J. Plant Physiol.* 17:73-81.

Dhindsa, R. S., Dong, G. & Lalonde, L. (1987). Altered gene expression during auxin-induced root development from excised mung bean seedlings. *Plant Physiol.* 84:1148-1153.

Drew, M. C. & Fourcy, A. (1986). Radial movement of cations across aerenchymatous roots of *Zea mays* measured by electron probe X-ray microanalysis. *J. Exp. Bot.* 37:823-831.

Druart, P., Kevers, C., Boxus, P. & Gaspar, T. (1982). *In vitro* promotion of root formation on apple shoots through darkness effect on endogenous phenolic and peroxidase. *Z. Pflanzenphysiol.* 108:429-436.

Dubois, P. (1978). *Plastics in Agriculture*. Applied Science. Barking, England.

Duke, S. O. & Vaughn, K. C. (1982). Lack of involvement of polyphenol oxidase in ortho-hydroxylation of phenolic compounds in mung bean seedlings. *Physiol. Plant.* 54:381-385.

Dunbar, B. S. (1987). *Two-Dimensional Electrophoresis and Immunological Techniques*. pp 77-102. Plenum Press. New York.

Dunberg, A., Hsihan, S. & Sandberg, G. (1981). Auxin dynamics and the rooting of cuttings of *Pinus sylvestris*. *Plant Physiol.* 67:5.

Duncan, R. & Hershey, J. W. B. (1984). Evaluation of isoelectric focusing running condition during two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis: variation of gel patterns with changing condition and optimized isoelectric focusing conditions. *Anal. Biochem.* 138:144-155.

Ehrlich, H. S. (1989). *PCR Technology: Principles and Applications for DNA Amplification.* pp 1-176. Stock Press. New York.

Eliasson, L. (1978). Effects of nutrients and light on growth and root formation in *Pisum sativum* cuttings. *Physiol. Plant.* 43:13-18.

Eliasson, L. & Brunes, L. (1980). Light effects on root formation in aspen and willow cuttings. *Physiol. Plant.* 48:261-265.

Endo, T. (1968). Indoleacetate oxidase activity of horseradish and other plant peroxidase isozymes. *Plant Cell Physiol.* 9:333-341.

Epstein, E. & Lavees, S. (1984). Conversion of IBA to IAA by cuttings of grapevine (*Vitis vinifera*) and olive (*Olea europea*). *Plant Cell Physiol.* 25:697-703.

Eriksen, E. N. (1974). Root formation in pea cuttings. III. The influence of cytokinin at different developmental stages. *Physiol. Plant.* 30:163-167.

Esau, K. (1953). *Plant Anatomy.* John Wiley & Sons. New York.

Fabbri, A., Lambardi, M. & Sani, P. (1985). The anatomy of adventitious root formation in cuttings of 140 Ruggeri (*Vitis berlandieri* × *Vitis rupestris*). *Riv. Vitic. Enol.* 38:508-515.

Fabijan, D., Taylor, J. S. & Reid, D. M. (1981). Adventitious rooting in hypocotyls of sunflower (Helianthus annuus) seedlings. II. Action of gibberellins, cytokinins, auxin and ethylene. *Physiol. Plant.* 53:589-597.

Fadl, M. S., Sari El-Deen, S. A. & El-mahdy, M. A. (1979). Physiological and chemical factors controlling adventitious root initiation in carob *Ceratonia siligua* L. stem cuttings. *Egyp. J. Hort.* 6:55-68.

Falasca, G., Reverberi, M., Lauri, P., Caboni, E., De Stradis, A. & Altamura, M. M. (2000). How *Agrobacterium rhizogenes* triggers *de novo* root formation in a recalcitrant woody plant: an integrated histological, ultrastructural and molecular analysis. *New Phytologist* 145:77-93.

Farquhar, G. D. & Sharkey, T. D. (1982). Stomatal conductance and photosynthesis. *Ann. Rev. Plant Physiol.* 33:317-345.

Featonby-Smith, B. C. & Van Staden, J. (1981). Endogenous cytokinins and rooting of leaf cuttings of *Phaseolus vulgaris* L. *Z. Pflanzenphysiol.* 102:329-335.

Fernqvist, I. (1966). Studies on factors in adventitious root formation. *Lantbruskhögskolans Annaler* 32:109-244.

Field, J. E. (1934). Experimental growing of insignis pine from slips. *N.Z. J. For.* 3:185-186.

Fielding, J. M. (1954). Methods of raising Monterey pine from cuttings in the open nursery. *Aust. For. & Timb. Bur. Bull.* 32:29.

Fielding, J. M. (1964). The possibility of using cuttings for the establishment of commercial plantations of Monterey pine. *Proc. World Consultation For. Gen. & Tree Impt.* p 7. Stockholm (FAO).

Fladung, M. (1990). Transformation of diploid and tetraploid potato clones with the *rolC* gene of *Agrobacterium rhizogenes* and charaterization of transgenic plants. *Plant Breeding* 104:295-304.

Foote, H. C. C., Brady, G., Thorlby, G. J. & Franklin, C. H. (1997). Subtractive hybridization of different mRNA populations. In Clark, M. S., ed. *Plant Molecular Biology - A Laboratory Mannual.* pp 201-220. Springer-Verlag. New York.

Franclet, A. (1979). Rajenunissement des arbres adults en vue de leur propagation végétative. In . *Micropropagation d'Arbres Forestiers.* pp 3-18. Assoc. Forêt-Cellulose. Nangis, France.

Frenkel, C. & Hess, C. E. (1974). Isozymic changes in relation to root initiation in mung bean. *Can. J. Bot.* 52:295-297.

Friedman, R., Altman, A. & Bachrach, U. (1982). Polyamines and root formation in mung bean hypocotyl cuttings. I. Effects of exogenous compounds and changes in endogenous polyamine content. *Plant Physiol.* 70:844-848.

Frolich, E. F. (1961). Etiolation and the rooting of cuttings. *Proc. Int. Plant Prop. Soc.* 11:277-283.

Fry, S. C. (1987a). Formation of isodityrosine by peroxidase isoenzymes. *J. Exp. Bot.* 38:853-862.

Fry, S. C. (1987b). Intracellular feruloylation of pectic polysaccharides. *Planta* 171:205-211.

Fukuda, H. & Komamine, A. (1982). Lignin synthesis and its related enzymes as markers of tracheary-elements differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Planta* 155:423-430.

Galston, A. W. & Kaur-Sawhney, R. (1987). Polyamines as endogenous growth regulator. In Davies, P. J., ed. *Plant Hormones and their Role in Plant Growth and Development.* pp 280-295. Martinus Nijhoff. Dordrecht.

García-Gómez, M. L., Sánchez-Romero, C., Barceló-Muñoz, A., Heredia, A. & Pliego-Alfaro, F. (1995). Peroxidase activity during adventitious root formation in avocado microcuttings. *Can. J. Bot.* 73:1522-1526.

García-Gómez, M. L., Sánchez-Romero, C., Barceló-Muñoz, A. & Pliego-Alfaro, A. H. F. (1994). Levels of endogenous indole-3-acetic acid and indole-3-aspartic acid during adventitious rooting in avocado microcuttings. *J. Exp. Bot.* 45:865-870.

Gardner, F. E. (1929). The relationship between the age and the rooting of cuttings. *Proc. Amer. Soc. Hort. Sci.* 26:101-104.

Gaspar, T. & Coumans, M. (1987). Root formation. In Bonga, J. M. & Durzan, D. J., eds. *Cell and Tissue Culture in Forestry.* pp 202-217. Martinus Nijhoff Pub. Dordrecht.

Gaspar, T. & Hofinger, M. (1988). Auxin metabolism during adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 117-131. Dioscorides Press. Portland, Oregon.

Gaspar, T., Kevers, C., Hausman, J. F., Berthon, J. Y. & Ripetti, V. (1992). Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. *Agronomie* 12:757-765.

Gaspar, T., Moncousin, C. & Greppin, H. (1990). The place and role of exogenous and endogenous auxin in adventitious root formation. In Millet, B. & Greppin, H., eds. *Intra- and Inter-Cellular Communications in Plants - Reception - Transmission - Storage and Expression of Messages.* pp 125-139. INRA. Paris.

Gaspar, T., Penel, C., Federico, J. C. & Greppin, H. (1985a). A two step control of basic and acidic peroxidases and its significance for growth and development. *Physiol. Plant.* 64:418-423.

Gaspar, T., Penel, C. & Greppin, H. (1997). Do rooting induction and flowering evocation involve a similar interplay between indoleacetic acid, putrescine and peroxidases? In Greppin, H., Penel, C. & Simon, P., eds. *Travelling Shot on Plant Development.* pp 35-49. University of Geneva. Geneva.

Gaspar, T., Penel, C., Roduit, C., Moncousin, C. & Greppin, H. (1985b). The role of auxin level and sensitivity in floral induction. *Biol. Plant.* 27:325-329.

Gay, A. P. & Loach, K. (1977). Leaf conductance changes on leafy cuttings of *Cornus* and *Rhododendron* during propagation. *J. Hort. Sci.* 52:509-516.

Gebhardt, K. (1985). Self-rooted sour cherries *in vitro*: auxin effect on rooting and isoperoxidases. *Acta Hortic.* 169:341-349.

Geneve, R. L., Hackett, W. P. & Swanson, B. T. (1990). Ethylene production in debladed petioles from the juvenile and mature phases of English ivy in relation to adventitious root initiation. *J. Amer. Soc. Hort. Sci.* 115:123-127.

Geneve, R. L. & Heuser, C. W. (1983). The relationship between ethephon and auxin on adventitious root initiation in cuttings of *Vigna radiata*(L.) R. Wilcz. *J. Amer. Soc. Hort. Sci.* 108:330-333.

Geneve, R. L. & Kester, S. T. (1991). Polyamines and adventitious root formation in the juvenile and mature phase of English ivy. *J. Exp. Bot.* 42:71-75.

Gianfagna, T. J. (1987). Natural and synthetic growth regulators and their use in horticultural and agronomic crops. In Davies, P. J., ed. *Plant Hormones and their Role in Plant Growth and Development.* pp 614-635. Martinus Nijhoff. Dordrecht.

Girouard, R. M. (1967). Initiation and development of adventitious roots in stem cuttings of *Hedera helix.* Anatomical studies of the juvenile growth phase. *Can. J. Bot.* 45:1877-1882.

Glick, D. & Nayyar, S. N. (1956). Studies in histochemistry: XLII further studies on the determination of succinic dehydrogenase in microgram amounts of tissue and distribution of the activity in the bovine adrenal. *J. Histochem. Cytochem.* 4:389-396.

Goldberg, R., Lê, T. & Catesson, A. M. (1985). Localization and properties of cell wall enzyme activities related to the final stage of lignin biosynthesis. *J. Exp. Bot.* 36:503-510.

González, A., Tamés, R. S. & Rodríguez, R. (1991). Ethylene in relation to protein, peroxidase and polyphenol oxidase activities during rooting in hazelnut cotyledons. *Physiol. Plant.* 83:611-620.

Good, G. L. & Tukey, H. B., Jr. (1966). Leaching of metabolites from cuttings propagated under intermittent mist. *Proc. Amer. Soc. Hort. Sci.* 89:727-733.

Gordon, S. A. & Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiol.* 26:192-195.

Gresshoff, P. M. & Doy, C. H. (1972). Development and differentiation of hapoid *Lycopersicon esculentum* (tomato). *Planta* 107:161-170.

Guevara-Garcia, A., Herrera-Estrella, L. & Olmedo-Alvarez, G. (1997). Use of PCRs in Plant Molecular Biology. In Clark, M. S., ed. *Plant Molecular Biology - A Laboratory Mannual.* pp 54-74. Springer-Verlag. New York.

Gurumurti, K., Gupta, B. B. & Kumar, A. (1984). Hormone regulation of root formation. In Purohit, S. S., ed. *Hormonal Regulation of Plant Growth and Development.* pp 387-400. Agro. Bot. Bub. Bikaner, India.

Hachett, W. P. (1988). Donor plant maturation and adventitious root formation. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 11-28. Dioscorides Press. Portland, Oregon.

Hachett, W. P. & Murray, J. R. (1994). Research on adventitious rooting: where do we go from here? In Davis, T. D. & Haissig, B. E., eds. *Biology of Adventitious Root Formation.* pp 333-335. Plenum Press. New York.

Hackett, W. P. & Murray, J. R. (1993). Maturation and rejuvenation in woody species. In Ahuja, M. R., ed. *Micropropagation of Woody Plants.* pp 93-105. Kluwer Academic Publisher. London.

Hagége, D., Kever, C., Boucaud, J. & Gaspar, T. (1988). Activités peroxydasiques, production d'ethylene, lignification et limitation de croissance chez *Suaeda maritima* cultivé an l'absence de NaCl. *Plant Physiol. Biochem.* 26:609-614.

Hagen, G., Uhrhammer, N. & Guilfoyle, T. J. (1988). Regulation of expression of an auxin-induced soybean sequence by cadminum. *J. Biol. Chem.* 263:6442-6446.

Haissig, B. E. (1970). Preformed adventitious root initiation in brittle willows grown in controlled environment. *Can. J. Bot.* 48:2309-2312.

Haissig, B. E. (1972). Meristematic activity during adventitious root primordium development. *Plant Physiol.* 49:886-892.

Haissig, B. E. (1974a). Influences of auxin and auxin synergists on adventitious root primordium initiation and development. *N.Z. J. For. Sci.* 4:311-323.

Haissig, B. E. (1974b). Metabolism during adventitious root primordium initiation and development. *N.Z. J. For. Sci.* 4:324-337.

Haissig, B. E. (1974c). Origins of adventitious roots. *N.Z. J. For. Sci.* 4:299-310.

Haissig, B. E. (1982a). Activity of some glycolytic and pentose phosphate pathway enzymes during the development of adventitious roots. *Physiol. Plant.* 55:261-272.

Haissig, B. E. (1982b). Carbohydrate and amino acid concentrations during adventitious root primordium development in *Pinus banksiana* Lamb. cuttings. *For. Sci.* 28:813-821.

Haissig, B. E. (1986). Metabolic processes in adventitious rooting of cuttings. In Jackson, M. B., ed. *New Root Formation in Plants and Cuttings.* pp 141-189. Martinus Niijhoff Publishers. Dordrecht/Boston/Lancaster.

Haissig, B. E. (1988). Future directions in adventitious rooting research. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 303-310. Dioscorides Press. Portland, Oregon.

Haissig, B. E., Davis, T. D. & Riemenschneider, D. E. (1992). Researching the controls of adventitious rooting. *Physiol. Plant.* 84:310-317.

Hammett, K. (1992). *Plant Propagation.* Second Edition. Reed Books, Octopus Publishing Group (NZ) Ltd. Auckland.

Hand, P. (1994). Biochemical and molecular markers of cellular competence for adventitious rooting. In Davis, T. D. & Haissig, B. E., eds. *Biology of Adventitious Root Formation.* pp 111-121. Plenum Press. New York.

Hansen, J. (1975). *Adventitious Root Formation - Influence of Light During Stock plant Growth.* The Royal Vet. and Agric. Univ. Internal review article. Copenhagen.

Hansen, J. (1988). Influence of gibberellins on adventitious root formation. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 162-173. Dioscorides Press. Porland, Oregon, USA.

Hansen, J., Strömquist, L. H. & Ericsson, A. (1978). Influence of the irradiance on carbohydrate content and rooting of cuttings on pine seedlings (*Pinus sylvestris* L.). *Physiol. Plant.* 61:975-979.

Harbage, J. F. & Stimart, D. P. (1996a). Effect of pH and 1*H*-indole-3-butyric acid (IBA) on rooting of apple microcuttings. *J. Amer. Soc. Hort. Sci.* 121:1049-1053.

Harbage, J. F. & Stimart, D. P. (1996b). Ethylene does not promote adventitious root formation on apple microcuttings. *J. Amer. Soc. Hort. Sci.* 121:880-885.

Harbage, J. F., Stimart, D. P. & Evert, R. F. (1993). Anatomy of adventitious root formation in microcuttings of *Malus domestica* Borkh. 'Gala'. *J. Amer. Soc. Hort. Sci.* 118:680-688.

Hartmann, H. T. & Kester, D. E. (1983). *Plant Propagation: Principles and Practices.* 4th Edition. pp 234-297. Prentice Hall, Englewood Cliffs. New Jersey, USA.

Hartung, W., Ohl, B. & Kummer, V. (1980). Abscisic acid and the rooting of runner bean cuttings. *Z. Pflanzenphysiol.* 98:95-103.

Hatcher, E. J. S. (1959). The propagation of rootstocks from stem cuttings. *Ann. Appl. Biol.* 47:635-639.

Hatta, M., Beyl, C. A., Garton, S. & Diner, A. M. (1996). Induction of roots on jujube softwood cuttings using *Agrobacterium rhizogenes*. *J. Hort. Sci.* 71:881-886.

Heide, O. M. (1964). Effects of light and temperature on the regeneration ability of *Begonia* leaf cuttings. *Physiol. Plant.* 17:789-804.

Heide, O. M. (1965). Photoperiodic effects on the regeneration ability of *Begonia* leaf cuttings. *Physiol. Plant.* 18:185-193.

Heide, O. M. (1968). Stimulation of adventitious bud formation in begonia leaves by abscisic acid. *Nature (London)* 219:960-961.

Herman, D. E. & Hess, C. E. (1963). The effect of etiolation upon the rooting of cuttings. *Proc. Int. Plant Prop. Soc.* 13:42-62.

Hess, C. E. (1969). Internal and external factors regulating root initiation. In Whittington, W. J., ed. *Root Growth.* pp 42-64. Butter Worths. London.

Heybroek, H. H. & Visser, T. (1976). Juvenility in fruit growing and forestry. *Acta Hortic.* 56:71-80.

Hicks, G. S. (1987). Adventitious rooting of apple microcuttings *in vitro*: an anatomical study. *Can. J. Bot.* 65:1913-1920.

Hinman, R. L. & Lang, J. (1965). Peroxidase catalyzed oxidation from ripening tomatoes. *Biochem.* 4:144-148.

Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A.-C., Miller, M. J. & Merril, C. R. (1988a). Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* 173:424-435.

Hochstrasser, D. F., Patchornik, A. & Merril, C. R. (1988b). Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining. *Anal. Biochem.* 173:412-423.

Hoffman, A. & Kummerow, J. (1966). Anatomische Beobachtungen zur Bewurzelung der Kurtstriebe von *Pinus radiata*. *Silvae Genetica* 15:35-38.

Horgan, K. & Aitken, J. (1981). Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. *Physiol. Plant.* 53:170-175.

Horgan, K. & Holland, L. (1989). Rooting micropropagated shoots from mature radiata pine. *Can. J. For. Res.* 19:1309-1315.

Huang, A. E. & Haard, N. F. (1977). Properties of IAA oxidase from ripening tomato fruit. *J. Food Biochem.* 1:93-110.

Humhpries, E. C. (1960). Inhibition of root development on petioles and hypocotyls of dwarf bean (*Phaseolus vulgaris*) by kinetin. *Physiol. Plant.* 13:659-663.

Hunter, C. S. (1979). *In vitro* culture of *Cinchona ledgeriana* L. *J. Hort. Sci.* 54:111-114.

Hutchison, K. W., Singer, P. B., McInnis, S., Diaz-Salaz, C. & Greenwood, M. S. (1999). Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin. *Plant Physiol.* 120:827-831.

Imberty, A., Goldberg, R. & Catesson, A. M. (1985). Isolation and characterization of *Populus isoperoxidases* involved in the last step of lignin formation. *Planta* 164:221-226.

Jackson, M. B. & Harney, P. M. (1970). Rooting cofactors, indoleacetic acid and adventitious root initiation in mung bean cuttings, (*Phaseolus aureus*). *Can. J. Bot.* 48:943-946.

Jacobs, M. R. (1939). The vegetative reproduction of forest trees. I. Experiments with cuttings of *P. radiata* Don. *Comm. For. Bur. (Canberra) Bull.* 25:28.

James, D. J. & Thurbon, I. J. (1981a). Phenolic compounds and other factors controlling rhizogenesis *In vitro* in the apple rootstocks M.9 and M.26. *Z. Pflanzenphysiol.* 105:11-20.

James, D. J. & Thurbon, I. J. (1981b). Shoot and root initiation *in vitro* in the apple rootstock M.9 and the promotive effects of phloroglucinol. *J. Hort. Sci.* 56:15-20.

Jarvis, B. C. (1986). Endogenous control of adventitious rooting in non-woody cuttings. In Jackson, M. B., ed. *New Root Formation in Plants and Cuttings.* pp 191-221. Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster.

Jarvis, B. C., Ali, A. H. N. & Shaheed, A. I. (1983a). Auxin and boron in relation to the rooting response and aging of mung bean cuttings. *New Phytol.* 95:509-518.

Jarvis, B. C. & Shaheed, A. I. (1986). Adventitious root formation in relation to the uptake and distribution of supplied auxin. *New Phytol.* 103:23-31.

Jarvis, B. C., Shannon, P. R. M. & Yasmin, S. (1983b). Involvement of polyamines with adventitious root development in stem cuttings of mung bean. *Plant Cell Physiol.* 24:677-683.

Jásik, J. & De Klerk, G.-J. (1997). Anatomical and ultrastructural examination of adventitious root formation in stem slices of apple. *Biol. Plant.* 39:79-90.

Jaunin, F., Rouelle-Rossier, V. B. & Hofer, R.-M. (1991). Cytochemical and X-ray microanalytical studies of calcium in the extending zone, particularly in the rhizodermis of *Zea mays* roots. *Physiol. Plant.* 81:15-23.

Jefferson, R. A., Burgess, S. M. & Hirsh, D. (1986). β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* 83:8447-8451.

Jermyn, M. A. (1975). Increasing the sensitivity of the anthrone method for carbohydrate. *Anal. Biochem.* 68:332-335.

Jones, O. P. & Hopgood, M. E. (1979). The successful propagation *in vitro* of two rootstocks of *Prunus*: the plum rootstock Pixy (*P. insititia*) and the cherry rootstock F12/1 (*P. avium*). *J. Hort. Sci.* 54:63-66.

Jusaitis, M. (1986). Rooting of intact mung bean hypocotyls stimulated by auxin, ACC, and low temperature. *HortScience* 21:1024-1025.

Kaminek, M. (1962). Prspeek fyziologii otuzovani rostlin. Zakarenovani rostlinnych Rizkv. *Rostlin. Vyrd.* 8:959-978.

Kaminski, C. (1971). Variations des activités peroxydasique et phénoloxydasique au cours de la croissance de *Coleus blumeni* Benth. var Automne. *Planta* 99:63-72.

Kang, M. S. (1995). Regulation of gene expression by 2,4-D during organogenesis in gladiolus cv. Topaz callus. *Acta Hortic.* 392:251-256.

Kantharaj, G. R., Mahadevan, S. & Padmanaban, G. (1979). Early biochemical events during adventitious root initiation in the hypocotyl of *Phaseolus vulgaris*. *Phytochem.* 18:383-387.

Kantharaj, G. R. & Padmanabhan, G. (1990). Molecular aspects of cytokinin's stymied action on auxin mediated new root formation in the hypocotyls of *Phaseolus vulgaris* L. Pages 131-139 in Prakash, J. & Pierik, R. L. M., eds. *Horticulture - New Technologies and Applications*. Kluwer Academic Publishers, Bangalor.

Kelly, A. J., Zagotta, M. T., White, R. A., Change, C. & Meeks-Wagner, D. R. (1990). Identification of genes expression in the tobacco shoot apex during the floral transition. *Plant Cell* 2:963-972.

King, J. J. & Stimart, D. P. (1998). Genetic analysis of variation for auxin-induced adventitious root formation among eighteen ecotypes of *Arabidopsis thaliana* L. Heynh. *J. Heredity* 89:481-487.

Kraus, E. J. & Kraybill, H. R. (1918). *Vegetation and Reproduction with Special Reference to Tomato*. Oregon Agric. Coll. Exp. Sta. Bull. 149.

Krelle, E. & Libbert, E. (1969). Interactions between abscisic acid and gibberellic acid regarding root formation. *Flora* 160:299-300.

Kriesel, K. (1976). Activity of cytokinin-like substances in the development of buds, newly formed shoots and adventitious roots of willow cuttings. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* 14:299-302.

Krishnamoorthy, H. N. (1972). Effect of growth retardants and abscisic acid on the rooting of hypocotyl cuttings of musk melo (*Cucumis melo* cv. Kutana). *Biochem. Physiol. Pflanzen* 163:513-517.

Krul, W. R. (1968). Increased root initiation in pinto bean hypocotyls with 2,4-dinitrophenol. *Plant Physiol.* 43:439-441.

Kummerow, J. (1966). Vegetative propagation of Monterey pine by means of needle fasicles. *For. Sci.* 12:391-398.

Lamport, D. T. A. (1986). Roles for peroxidase in cell wall genesis. In Greppin, H., Penel, C. & Gaspar, T., eds. *Molecular and Physiological Aspects of Plant Peroxidases.* pp 199-208. University of Geneve. Geneve.

Land, H., Grez, M., Hauser, H., Lindenmaier, W. & Schütz, G. (1983). Synthesis of ds-cDNA involving addition of dCMP tails to allow cloning of 5'-terminal mRNA sequences. In Wu, R., Grossman, L. & Moldave, K., eds. *Methods in Enzymology.* pp 285-292. Academic Press, Inc. New York.

Leroy, P., Merlino, M., Nègre, S., Caissard, J. C., Sourdille, P., Lu, Y. H. & Bernard, M. (1997). Non-radioactive methods of detection on Southern Blots. In Clark, M. S., ed. *Plant Molecular Biology - A Laboratory Mannual.* pp 41-53. Springer-Verlag. New York.

Leshem, B. & Sussex, I. M. (1990). Polypeptides of cultured melon cotyledons served as markers of root and shoot organogenesis. *J. Plant Physiol.* 137:155-159.

Letham, D. S. & Palni, L. M. S. (1983). The biosynthesis and metabolism of cytokinins. *Ann. Rev. Plant Physiol.* 34:163-197.

Levesque, H., Delepelaire, P., Rouzé, P., Slightom, J. & Tepfer, D. (1988). Common evolutionary origin of the central portions of the Ri TL-DNA of *Agrobacterium rhizogenes* and the Ti-T-DNAs of *Agrobacterium tumefacines*. *Plant Mol. Biol.* 11:731-744.

Lewis, D. H. (1980). Are there inter-relations between the metabolic role of boron, synthesis of phenolic phytoalexins and germination of pollen? *New Phytol.* 84:261-270.

Libby, W. J., Jr, Brown, A. G. & Fielding, J. M. (1972). Effects of hedging radiata pine on production, rooting and early growth of cuttings. *N.Z. J. For. Sci.* 2:263-283.

Libby, W. J. & Conkle, M. T. (1966). Effects of auxin treatment, tree age, tree vigour, and cold storage on rooting young Monterey pine. *For. Sci.* 12:484-502.

Loach, K. (1988a). Controlling environmental conditions to improve adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 248-273. Dioscorides Press. Portland, Oregon.

Loach, K. (1988b). Water relations and adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* Dioscorides Press. Portland, Oregon.

Lockhart, J. A. (1955). The role of 2,4-dichlorophenol in the destruction of indoleacetic acid by peroxidase. *Plant Physiol.* 30:86-88.

Lovell, P. H., Illsley, A. & Moore, K. G. (1974). Endogenous sugar levels and their effects on root formation and petiole yellowing of detached mustard cotyledons. *Physiol. Plant.* 31:321-326.

Lovell, P. H. & White, J. (1986). Anatomical changes during adventitious root formation. In Jackson, M. B., ed. *New Root Formation in Plants and Cuttings.* pp 111-140. Martinus Nijhoff Publishers. Dordrecht/Boston/Lancaster.

Machida, H., Ooshi, A., Hosoi, T., Komatsu, H. & Kamota, F. (1977). Studies on photosynthesis in cuttings during propagation. I. Changes in the rate of apparent photosynthesis in the cuttings of several plants after planting. *J. Jap. Soc. Hortic. Sci.* 46:274-282.

Macisaac, S. A. & Sawhney, V. K. (1990). Protein changes associated with auxin-induced stimulation and kinetin-induced inhibition of lateral root initiation in lettuce (Lactuca sativa) roots. *J. Exp. Bot.* 41:1039-1044.

Maldivey, R., Pelese, F., Pilate, G., Sotta, B., Sossountzov, L. & Miginiac, E. (1986). Endogenous levels of abscisic acid, indole-3-acetic acid, zeatin and zeatinriboside during the course of adventitious root formation in cuttings of Craigella and Craigella lateral suppressor tomatoes. *Plant Physiol.* 68:426-430.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning - A Laboratory Manual.* Cold Spring Harbor Laboratory. New York.

Marchuk, D., Drumm, M., Saulino, A. & Collins, F. S. (1990). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Necl. Acids Res.* 19:1154.

Martin, B. & Quillet, G. (1974). Bouturage des arbres forestiers en Congo. Résultats des essais effectués à Pointe-Noire de 1969 à 1973. Rajeunissement des arbres plus et constitution du parc a bios. *Bois et Forê des Tropiques* 157:21-40.

Maurel, C., Barbier-Brygoo, H., Spena, A., Tempé, J. & Guern, J. (1991). Single *rol* genes from *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum. Plant Physiol.* 97:212-216.

Maurel, C., Leblanc, N., Barbier-Brygoo, H., Perrot-Rechenmann, C., Bouvier-Durand, M. & Guern, J. (1994). Time course of *rolB* mRNA expression and increase in auxin sensitivity reveal multiple control by auxin. *Plant Physiol.* 105:1209-1215.

Mayer, A. M. (1987). Polyphenol oxidases in plants - recent progress. *Phytochem.* 26:11-20.

Mayer, A. M. & Harel, E. (1979). Polyphenol oxidases in plants. *Phytochem.* 18:193-215.

Mayer, J. E., Hahne, G., Palme, K. & Schell, J. (1987). A simple and general plant tissue extraction procedure for two-dimensional gel electrophoresis. *Plant Cell Reports* 6:77-81.

Maynard, B. K. & Bassuk, N. L. (1988). Etiolation and banding effects on adventitious root formation. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 29-46. Dioscorides Press. Portland, Oregon.

McAfee, B. J., White, E. E., Pelcher, L. E. & Lapp, M. S. (1993). Root induction in pine (*Pinus*) and larch (*Larix*) pps. using *Agrobacterium rhizogenes*. *Plant Cell Tiss. Org. Cult.* 34:53-62.

McCready, R. M., Guggolz, J., Silviera, V. & Owens, H. S. (1950). Determination of starch and amylose in vegetables. *Anal. Chem.* 22:1156-1158.

McGee, J. D., Williams, E. G., Collins, G. B. & Hildebrand, D. F. (1989). Somatic embryogenesis in *Trifolium*: Protein profiles associated with high and low frequency regeneration. *J. Plant Physiol.* 135:306-312.

McQueen-Mason, S. (1995). Expansins and cell wall expansion. *J. Exp. Bot.* 46:1639-1650.

McQueen-Mason, S. J., Durachko, M. D. & Cosgrove, D. J. (1992). Two endogenous proteins that induce cell wall extension in plants. *Plant Cell* 4:1425-1433.

McVeigh, I. (1938). Regeneration in *Crsaaula multicava*. *Amer. J. Bot.* 25:7-11.

Meeks-Wagner, D. R., Dennis, E. S., Tran Thanh Van, K. & Peacock, W. J. (1989). Tobacco genes expressed during *in vitro* floral initiation and their expression during normal plant development. *Plant Cell* 1:25-35.

Melchior, G. H. (1963). Preliminary report on the vegetative propagation of *Pinus radiata* and *P. oocarpa*. *Bol. Ins. For. Latino America, Merida* No. 13:13-21.

Melzer, S., Majewski, D. M. & Apel, K. (1990). Early changes in gene expression during the transition from vegetative to generative growth in the long-day plant *Sinapis alba*. *Plant Cell* 2:953-961.

Mengel, K. & Kirkby, E. A. (1982). *Principles of Plant Nutrition.* 3rd Edition. Int. Potash Inst. Bern, Switzerland.

Michniewicz, M. & Kriesel, K. (1970). Dynamics of auxins, gibberellin-like substances and growth inhibitors in the rooting process of black poplar cuttings (*Populus nigra* L.). *Acta Soc. Bot. Pol.* 39:383-390.

Miller, A. R., Crawford, D. L. & Roberts, L. W. (1985). Lignification and xylogenesis in *Lactuca* pith explants cultured in vitro in the presence of auxin and cytokinin: A role for endogenous ethylene. *J. Exp. Bot.* 36:110-118.

Mirov, N. T. (1944). Experiments in rooting pines in California. *J. For* 42:199-204.

Mitsuhashi-Kato, M., Shibaoka, H. & Shimokoriyama, M. (1978a). Anatomical and physiological aspeacts of development processes of adventitious root formation in *Azukia* cuttings. *Plant Cell Physiol.* 19:393-400.

Mitsuhashi-Kato, M., Shibaoka, H. & Shimokoriyama, M. (1978b). The nature of the dual effect of auxin on root formation in *Azukia* cutting. *Plant Cell Physiol.* 19:1535-1542.

Moe, R. & Andersen, A. S. (1988). Stock plant environment and subsequent adventitious rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 214-234. Dioscorides Press. Portland, Oregon.

Molnar, J. M. & LaCroix, L. J. (1972a). Studies of the rooting of cuttings of *Hydrangea macrophylla*: DNA and protein changes. *Can. J. Bot.* 50:387-392.

Molnar, J. M. & LaCroix, L. J. (1972b). Studies of the rooting of cuttings of *Hydrangea macrophylla*: enzyme changes. *Can. J. Bot.* 50:315-322.

Moncousin, C., Favre, J. M. & Gaspar, T. (1988). Changes in peroxidase activity and endogenous IAA levels during adventitious rooting in vine cuttings. In Kutacek, M., Bandurski, R. S. & Krekule, J., eds. *Physiology and Biochemistry of Auxins in Plants.* pp 331-337. Academia. Prague.

Moncousin, C., Favre, J. M. & Gaspar, T. (1989). Early changes in auxin and ethylene production in vine cuttings before adventitious rooting. *Plant Cell Tiss. Org. Cult.* 19:235-242.

Moncousin, C. H. & Gaspar, T. (1983). Peroxidase as a marker for rooting improvement in *Cynara scolymus* L. cultured *in vitro*. *Biochem. Physiol. Pflanzen* 178:263-271.

Montain, C. R., Haissig, B. E. & Curtis, J. D. (1983a). Differentiation of adventitious root primordia in callus of *Pinus banksiana* seedlings cuttings. *Can. J. For. Res.* 13:195-200.

Montain, C. R., Haissig, B. E. & Curtis, J. D. (1983b). Initiation of adventitious root primordia in very young *Pinus banksiana* seedling cuttings. *Can. J. For. Res.* 13:191-195.

Moore, L. W., Warren, G. & Strobel, G. (1979). Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid* 2:617-626.

Mudge, K. W. (1988). Effect of ethylene on rooting. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 150-161. Dioscorides Press. Porland, Oregon, USA.

Mudge, K. W. & Swanson, B. T. (1978). Effect of ethephon, indole bytyric acid, and treatment solution pH on rooting and on ethylene levels within mung bean cuttings. *Plant Physiol.* 61:271-273.

Mullins, M. G. (1985). Regulation of adventitious root formation in microcuttings. *Acta Hortic.* 166:53-61.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.

Nanda, K. K. (1975). Physiology of adventitious root formation. *Ind. J. Plant Physiol.* 18:80-89.

Nanda, K. K. & Anand, V. K. (1970). Seasonal changes in auxin effects on rooting of stem cuttings of *Populus nigra* and its relationship with mobilization of starch. *Physiol. Plant.* 23:99-107.

Nanda, K. K., Bansal, G. L., Kochhar, V. K. & Bhattcharya, N. C. (1978). Effect of some metabolic inhibitors of oxidative phosphorylation on rooting of hypocotyl cuttings of *Phaseolus mungo*. *Ann. Bot.* 42:659-663.

Nanda, K. K., Bhattacharya, N. C. & Kaur, N. P. (1973). Effect of morphactin on peroxidases and its relationship with rooting hypocotyl cuttings on *Impatiens balsamina*. *Plant Cell Physiol.* 14:297-311.

Nanda, K. K., Bhattacharya, N. C. & Kochhar, V. K. (1974). Biochemical basis of adventitious root formation on etiolated stem segments. *N.Z. J. For. Sci.* 4:347-358.

Nanda, K. K. & Dhawan, A. K. (1976). A paradoxical effect of 2,4-dinitrophenol in rooting hypocotyl cuttings of *Phaseolus mungo*. *Experientia* 32:1167-1168.

Nanda, K. K., Gurumurti, K. & Chibbar, R. N. (1975). Evidence for the allosteric nature of IAA-oxidase system in *Phaseolus mungo* hypocotyl. *Experientia* 31:635-636.

Nanda, K. K., Purohit, A. N. & Bala, A. (1967). Effect of photoperiod, auxins and gibberrellic acid on rooting of stem cuttings of *Bryophyllum tubiflorum* Harv. *Physiol. Plant.* 20:1096-1102.

Nanda, K. K., Purohit, A. N. & Kochhar, V. K. (1969). Effect of auxins and light on rooting stem cuttings of *Populus nigra* and its relationship with mobilization of starch. *Physiol. Plant.* 23:99-107.

Naylor, E. & Johnson, B. (1937). A histological study of vegetative reproduction in *Saintpaulia ionantha*. *Amer. J. Bot.* 24:673-678.

Nichols, S. E. & Laties, G. G. (1984). Ethylene-regulated gene transcription in carrot roots. *Plant Mol. Biol.* 3:393-401.

Nilsson, O. (1995). *Effects of Agrobacterium rhizogenes rol-Gene Expression on Plant Growth and Hormone Metabolism*. SLU Reprocentralen. Umeå.

Nin, S., Bennici, A., Roselli, G., Mariotti, D., Schiff, S. & Magherini, R. (1997). *Agrobacterium*-mediated transformation of *Artemisia absinthium* L. (wormwood) and production of secondary metabolites. *Plant Cell Reports* 16:725-730.

Noiton, D., Vine, J. H. & Mullins, M. G. (1992). Endogenous indole-3-acetic acid and abscisic acid in apple microcuttings in relation to adventitious root formation. *Plant Growth Regul.* 11:63-67.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.

Okoro, O. O. & Grace, J. (1976). The physiology of rooting *Populus* cuttings. I. Carbohydrates and photosynthesis. *Physiol. Plant.* 36:133-138.

Oliver, M. J., Mukherjee, I. & Reid, D. M. (1994). Alteration in gene expression in hypocotyls of sunflower (*Helianthus annuus*) seedlings associated with derooting and formation of adventitious root primordia. *Physiol. Plant.* 90:481-489.

Palanisamy, K. & Kumar, P. (1997). Seasonal variation on adventitious rooting in branch cuttings of *Pongamia pinnata* Pierre. *Indi. For.* 123:236-239.

Pan, R. & Tian, X. S. (1999). Comparative effect of IBA, BSAA and 5,6-Cl-2-IAA-Me on the rooting of hypocotyl in mung bean. *Plant Growth Regul.* 27:91-98.

Pan, R. C. & Gui, H. (1997). Physiological basis of the synergistic effects of IBA and triadimefon on rooting of mung bean hypocotyls. *Plant Growth Regul.* 22:7-11.

Passey, A. J. & Jones, O. P. (1983). Shoot proliferation and rooting *in vitro* of *Theobroma cacao* L. type Amelonado. *J. Hort. Sci.* 58:589-592.

Patena, L., Sutter, E. G. & Dandekar, A. M. (1988). Root induction by *Agrobacterium rhizogenes* in a difficult-to-root woody species. *Acta Hortic.* 227:324-329.

Patience, P. A. & Alderson, P. G. (1987). Development of a system to study peroxidases during the rooting of lilac shoots produced *in vitro*. *Acta Hortic.* 212:267-272.

Paton, D. M., Nicholls, W. & Pryor, L. D. (1970). Rooting of stem cuttings of *Eucalyptus*: A rooting inhibitor in adult tissues. *Aust. J. Bot.* 18:175-183.

Pavlová, L. & Krekule, J. (1984). Fluctuation of free IAA under inductive and non-inductive photoperiods in Chenopodium rubrum. *Plant Growth Regul.* 2:91-98.

Pawsey, C. K. (1950). Some observations upon the vegetative reproduction of Monterey pine. *Aust. For.* 14:90-94.

Pedroso, M. C. & Pais, M. S. (1992). A scanning electron microscopy and X-ray microanalysis study during induction of morphogenesis in *Camellia japonica*. *Plant Sci.* 87:99-108.

Pedroso, M. C. & Pais, M. S. (1994). Early detection of embryogenic competence and polarity in *Camellia japonica* L. by electron probe X-ray microanalysis. *Plant Sci.* 96:189-201.

Pelese, F., Megnegneau, B., Sotta, B., Sossountzov, L., Caboche, M. & Miginiac, E. (1989). Hormonal characterization of a nonrooting naphthaleneacetic acid tolerant tobacco mutant by an immunoenzymic method. *Plant Physiol.* 89:86-92.

Peterson, T. A., Swanson, E. S. & Hull, R. J. (1986). Use of lanthanum to trace apoplastic solute transport in intact plants. *J. Exp. Bot.* 37:807-822.

Philips, I. D. J. (1964). Root-shoot hormone relations. II. Changes in endogenous auxin concentrationa produced by flooding of the root system in *Helianthus annuus*. *Ann. Bot.* 28:37-45.

Pingel, U. (1976). Der Einfluss phenolischer Aktivatoren und inhibitoren der IES-Oxydase-Aktivität auf die Adventivbewurzelung bei *Tradescantia albiflora*. *Z. Pflanzenphysiol.* 79:109-120.

Pitman, M. G., Läuchli, A. & Stelzer, R. (1981). Ion distribution in roots of barley seedlings measured by electron probe X-ray microanalysis. *Plant Physiol.* 68:673-679.

Poapst, P. A. & Durkee, A. B. (1967). Root-differentiating properties of some simple aromatic substances of the apple and pear fruit. *J. Hort. Sci.* 42:429-438.

Pomponi, M., Spanò, L., Sabbadini, M. G. & Costantino, P. (1983). Restriction endonuclease mapping of the root-inducing plasmid of *Agrobacterium rhizogenes* 1855. *Plasmid* 10:119-129.

Porlingis, I. C. & Koukourikou-Petridou, M. (1996). Promotion of adventitious root formation in mung bean cuttings by four triazole growth retardants. *J. Hort. Sci.* 71:573-579.

Porter, J. R. (1991). Host range and implications of plant infection by *Agrobacterium rhizogenes*. *Crit. Rev. Plant Sci.* 10:387-421.

Pressey, R. (1990). Anions activated the oxidation of indoleacetic by peroxidases from tomato and other sources. *Plant Physiol.* 93:798-804.

Pythoud, F. & Buchala, A. J. (1989). Peroxidase activity and adventitious rooting in cuttings of *Populus tremula*. *Plant Physiol. Biochem.* 27:503-510.

Rajagopal, V., Rao, M. R. K. & Rao, I. M. (1971). Influence of indoleacetic acid and abscisic acid on the rooting of tomato shoot cuttings. *Ind. J. Plant Physiol.* 14:91-96.

Ramagli, L. S. & Rodriguez, L. V. (1985). Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6:559-563.

Rancillac, M., Faye, M. & David, A. (1982). In vitro rooting of cloned shoots in *Pinus pinaster*. *Physiol. Plant.* 56:97-101.

Rasmussen, S. & Andersen, A. S. (1980). Water stress and root formation in pea cuttings. II. Effect of abscisic acid treatment of cuttings from stock plants grown under two levels of irradiance. *Physiol. Plant.* 48:150-154.

Rayle, D. L. & Cleland, R. E. (1992). The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* 99:1271-1274.

Read, P. E. & Hoysler, V. C. (1971). Improving rooting of carnation and poinsettia cuttings with succinic acid-2,2-dimethylhyhydrazide. *HortScience* 6:350-351.

Reid, M. S. (1987). Ethylene in plant growth, development, and senescence. In Davies, P. J., ed. *Plant Hormones and their Role in Plant Growth and Development.* pp 257-279. Martinus Nijhoff. Dordrecht.

Reilly, K. & Washer, J. (1977). Vegetative propagation of radiata pine by tissue culture: plantlet formation from embryonic tissue. *N.Z. J. For. Sci.* 7:199-206.

Riker, A. J. (1930). Studies on infectious hairy root of nursery apple trees. *J. Agric. Res.* 4:507-540.

Riov, J. & Yang, S. F. (1989). Ethylene and auxin-ethylene interaction in adventitious root formation in mung bean cuttings. *J. Plant Growth Regul.* 8:131-141.

Robbins, J. A., Kays, S. J. & Dirr, M. A. (1983). Enhanced rooting of wounded mung bean cuttings by wounding and ethephon. *J. Amer. Soc. Hort. Sci.* 108:325-329.

Robbins, J. A., Reid, M. S. & Rost, T. L. (1985). The effect of ethylene on adventitious root formation in mung bean (*Vigna radiata*) cuttings. *J. Plant Growth Regul.* 4:147-157.

Rodriguez, A., Albuerne, M. & Sanchez-Tames, R. (1988). Rooting ability of *Corylus avellanaI L.*: macromorphological and histological study. *Scientia Hortic.* 35:131-142.

Rout, G. R., Samantaray, S. & Das, P. (2000). *In vitro* rooting of *Psoralea corylifolia* Linn: Peroxidase activity as a marker. *Plant Growth Regul.* 30:215-219.

Rozema, L. (1979). Population dynamics and ecophysiological adaptions of some coastal members of the *Juncaceae* and *Gramineae*. In Jefferies, R. L. & Davy, A. J., eds. *Ecological Processes in Coastal Environments.* pp 229-241. Blackwell Scientific Publications. Oxford.

Rugini, E. & Fedeli, E. (1990). Olive (*Olea europaea* L.) as an oilseed crop. In Bajaj, Y. P. S., ed. *Biotechnology in Agriculture and Forestry.* pp 593-641. Springer-Verlag. Berlin Heidelberg, New York.

Rugini, E., Jacoboni, A. & Luppino, M. (1993). Role of basal shoot darkening and exogenous putrescine treatments on in vitro rooting and on endogenous polyamine changes in difficult-to-root woody species. *Scientia Hortic.* 53:63-72.

Rugini, E., Luppino, M., De Agazio, M. & Grego, S. (1991). Endogenous polyamine and root morphogenesis variations under different treatments in cuttings and in *in vitro* explants of olive. *Acta Hortic.* 300:225-232.

Rugini, E., Politi, V., Bignami, C., De Agazio, M. & Grego, S. (1990). Effect of polyamine treatment on rooting of three olive cultivar cutting. *Acta Hortic.* 286:97-100.

Sankhla, N. & Upadhyaya, A. (1988). Polyamines and adventitious root formation. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 202-213. Dioscorides Press. Portland, Oregon.

Satoo, S. (1952). Origin and development of adventitious roots in seedling cuttings of conifers. I. *Bull. Tokyo Univ. For.* 43:59-81.

Satoo, S. (1955). Origin and development of adventitious roots in seedling cuttings of conifers. II. *Bull. Tokyo Univ. For.* 48:115-128.

Satoo, S. (1956). Anatomical studies on the rooting of cuttings in coniferous species. *Bull. Tokyo Univ. For.* 51:109-158.

Schmidt, G. (1982). Different methods of etiolation for increasing the rooting of the Silver Lime, *Tilia tomentosa*, softwood cuttings. *Proc. Int. Soc. for Hortic. Sci. 21st Int. Hortic. Cong.*

Schmülling, T., Fladung, M., Grossmann, K. & Schell, J. (1993). Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. *Plant J.* 3:371-382.

Schmülling, T., Schell, J. & Spena, A. (1988). Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J.* 7:2621-2629.

Selby, C., Kennedy, S. J. & Harvey, B. M. R. (1992). Adventitious root formation in hypocotyl cuttings of *Picea aitchensis* (Bong) Carr. -- the influence of plant growth regulators. *New Phytol.* 120:453-457.

Setterfield, G., Sparkuhl, J. & Bryne, H. (1978). Ribosome metabolism in excised slices of Jerusalem artichoke tuber. In Kahl, G., ed. *Biochemistry of Wounded Plant Tissue*. W. de Gruyter. Berlin.

Shapiro, S. (1958). The role of light in the growth of root primordia in the stems of lambardy poplar. In Thimann, K. V., ed. *Physiology of Forest Trees*. Ronald Press Co. New York.

Shcherban, T. Y., Shi, J., Durachko, M. D., Guiltinan, M. J., McQueen-Mason, S. J., Sheih, M. & Cosgrove, D. J. (1995). Molecular cloning and sequence analysis of expansins: a high conserved, multigene family of proteins that mediates cell wall extension in plants. *Proc. Natl. Acad. Sci. USA* 92:9245-9249.

Shen, X. S. & Mullins, M. G. (1983). *In vitro* propagation of pear *Pyrus communis* L. cvs. Packham's Triumph, William's 'Bon Chretien and Beurre' Bosc. *Scientia Hortic.* 23:51-57.

Sherry, S. P. (1942). A note on the vegetative propagation of *Pinus insignis*. *J. S. Afr. For. Assoc.* 9:23-25.

Shimokawa, K. (1983). An ethylene-forming enzyme in Citrus unshiu fruits. *Phytochem.* 22:1903-1908.

Shyr, Y. & Kao, C. (1985). Polyamines and root formation in mung bean hypocotyl cuttings. *Bot. Bull. Academia Sinica* 26:179-184.

Simpson, G. M. & Naylor, J. M. (1962). Dormancy studies in seed of *Avena fatua.* 3. A relationship between maltase, amylase, and gibberellin. *Can. J. Bot.* 40:1659-1674.

Simpson, R. B., Spielmann, A., Margossian, L. & McKnight, T. (1986). A disarmed binary vector from *Agrobacterium tumefacines* function in *Agrobacterium rhizogenes*. *Plant Mol. Biol.* 6:403-415.

Sinkar, V. P., Pythoud, F., White, F. F., Nester, E. W. & Gordon, M. P. (1988). *rolA* locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. *Genes Dev.* 2:688-697.

Smith, B. J. (1984). SDS polyacrylamide gel electrophoresis of proteins. In Walker, J. M., ed. *Methods in Molecular Biology.* pp 41-61. Humana Press. Clifton, New Jersey.

Smith, D. L. & Fedoroff, N. V. (1995). *LRP1*, a gene expressed in lateral and adventitious root primordia of *Arabidopsis*. *Plant Cell* 7:735-745.

Smith, D. R. & Thorpe, T. A. (1975a). Root initiation in cuttings of *Pinus radiata* seedlings. I. Developmental sequence. *J. Exp. Bot.* 26:184-192.

Smith, D. R. & Thorpe, T. A. (1975b). Root initiation in cuttings of *Pinus radiata* seedlings. II. Growth regulator interactions. *J. Exp. Bot.* 26:193-202.

Smith, D. R. & Thorpe, T. A. (1977). Root initiation in cuttings of *Pinus radiata* seedlings: Effects of aromatic amino acids and simple phenylpropanoids. *Bot. Gaz.* 138:434-437.

Smith, D. R. & Wareing, P. F. (1972a). The rooting of actively growing and dormant leafy cuttings in relation to the endogenous hormone levels and photoperiod. *New Phytologist* 71:483-500.

Smith, D. R. & Wareing, P. F. (1972b). Rooting of hardwood-cuttings in relation to bud dormancy and the auxin content of the excised stems. *New Phytologist* 71:63-80.

Smulders, M. J. M., van de Ven, E. T. W. M., Croes, A. F. & Wullems, G. J. (1990). Metablism of 1-naphthaleneacetic acid in explants of tobacco: evidence for relaese of free hormone from conjugates. *J. Plant Growth Regul.* 9:27-34.

Spanò, L. & Costantino, P. (1982). Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on tobacco. *Z. Pflanzenphysiol.* Bd. 106:87-92.

Spena, A., Schmülling, T., Koncz, C. & Schell, J. (1987). Independent and synergistic activity of *rolA, B and C* loci in stimulating abnormal growth in plants. *EMBO J.* 6:3891-3899.

Sponsel, V. M. (1985). Gibberellins in *Pisum sativum* -- their nature, distribution and involvement in growth and development of plant. *Physiol. Plant.* 65:533-538.

Stafstrom, J. P. & Sussex, I. M. (1988). Patterns of protein synthesis in dormant and growing vegetative buds of pea. *Planta* 176:497-505.

Stenlid, G. (1963). The effect of flavonoid compounds on oxidative phosphorylation and on the enzymatic destruction on indole-acetic acid. *Physiol. Plant.* 16:110-120.

Stoltz, L. P. (1968). Factors influencing root initiation in an easy- and a difficult-to-root *Chrysanthemum*. *Proc. Amer. Soc. Hort. Sci.* 92:622-626.

Stoutemyer, V. T. (1937). Regeneration in various types of applewood. *Iowa Agric. Exp. Bull.* 220:307-352.

Strack, D., Ruhoff, R. & Gräwe, W. (1986). Hydroxycinnamoyl-coenzyme-a: tartronate hydroxycinnamoyltransferase in protein preparations from mung bean. *Phytochem.* 25:833-837.

Strobel, G. A. & Nachmias, A. (1985). *Agrobacterium rhizogenes* promotes the initial growth of bare root stock almond. *J. Gen. Microbiol.* 131:1245-1249.

Strobel, G. A. & Nachmias, A. (1988). *Agrobacterium rhizogenes*: A root inducing bacterium. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious root formation in cuttings.* pp 284-288. Dioscorides Press. Portland, Oregon.

Strobel, G. A., Nachmias, A. & Hess, W. M. (1988). Improvements in the growth and yield of olive trees by transformation with Ri plasmid of *Agrobacterium rhizogenes*. *Can. J. Bot.* 66:2581-2585.

Strydom, D. K. & Hartmann, H. T. (1960). Effect of indolebutyric acid on respiration and nitrogen metabolism in Marianna 2624 plum softwood stem cuttings. *Proc. Amer. Soc. Hort. Sci.* 76:124-133.

Stuart, N. W. (1938). Nitrogen and carbohydrate metabolism of kidney bean cuttings as affected by treatment with indoleacetic acid. *Bot. Gaz.* 100:298-311.

Sussex, I. M., Godoy, J. A., Kerk, N. M., Laskowski, M. J., Nusbaum, H. C., Welsch, J. A. & Williums, M. (1995). Cellular and molecular events in a newly organizing lateral root meristem. *Phil. Trans. R. Soc. Lond. B* 350:39-43.

Svenson, S. E. & Davies, F. T., Jr. (1995). Changes in tissue mineral elemental concentration during root initiation and development of poinsettia cuttings. *HortScience* 30:617-619.

Takeno, K., Taylor, J. S., S Sriskandarajah, Pharis, R. P. & Mullins, M. G. (1982/83). Endogenous gibberellin- and cytokinin-like substances in cultured shoot tissues of apple., *Malus pumila* cv. Jonathan, in relation to adventitious root formation. *Plant Growth Regul.* 1:261-268.

Tari, I. & Nagy, M. (1996). Abscisic acid and Ethrel abolish in inhibition of adventitious root formation of paclobutrazol treated bean primary leaf cuttings. *Biol. Plant.* 38:369-375.

Taylor, B. H. & Scheuring, C. F. (1994). A molecular marker for lateral root initiation: The RSI-1 gene of tomato (*Lycopersicon esculentum* Mill.) is activated in early lateral root primordia. *Mol. Gen. Genet.* 243:148-157.

Thorpe, T. A., Than Van, M. T. & Gaspar, T. (1978). Isoperoxidase in epidermal layers of tobacco and changes during organ formation *in vitro*. *Physiol. Plant.* 44:388-394.

Thulin, I. J. & Faulds, T. (1968). The use of cuttings in the breeding and afforestation of *Pinus radiata*. *N.Z. J. For.* 13:66-77.

Tiburcio, A. F., Gendy, C. A. & Tran Than van, K. (1989). Morphogenesis in tobacco subepidermal cells: putrescine as marker of root differentiation. *Plant Cell Tiss. Org. Cult.* 19:43.

Tomaszewski, M. & Thimann, K. V. (1966). Interaction of phenolic acid, metallic ions and chelating agents on auxin-induced growth. *Physiol. Plant.* 41:1443-1454.

Towner, P. (1991a). Purification of DNA. In Brown, T. A., ed. *Essential Molecular Biology - A Practical Approach.* pp 47-68. Oxford University Press. New York.

Towner, P. (1991b). Recover of DNA from electrophoresis gels. In Brown, T. A., ed. *Essential Molecular Biology - A Practical Approach.* pp 127-141. Oxford University Press. New York.

Travis, G. H. & Sutcliffe, J. G. (1988). Phenol emulsion-enhanced DNA-driven subtractive cDNA cloning: isolation of low-abundance monkey cortex-specific mRNAs. *Proc. Natl. Acad. Sci. USA* 85:16996-11700.

Tripepi, R. R., Heuser, C. W. & Shannon, J. C. (1983). Incorporation of tritiated thymidine and uridine into adventitious-root initial cells of *Vigna radiata*. *J. Amer. Soc. Hort. Sci.* 108:469-474.

Turetskaya, R. K. & Kof, E. M. (1965). Dynamics in the changes in auxins and inhibitors in green and etiolated bean cuttings in the process of root formation. *Dokl. Bot. Sci.* 165:118-121.

Tzfira, T., Ben-Meir, H., Vainstein, A. & Altman, A. (1996a). High efficient transformation and regeneration of aspen plants through shoot-bud formation in root culture. *Plant Cell Reports* 15:566-571.

Tzfira, T., yarnitzky, O., Vainstein, A. & Altman. (1996b). *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis* Mill. *Plant Cell Reports* 16:26-31.

Upadhyaya, A., Davis, T. D., Sankhla, D., Sankhla, N. & Smith, B. N. (1985). Effect of paclobutrazol on the activities of proteolytic enzymes and RNase in soybean leaves during senescence. *Comparative Physiol. Ecol.* 10:49-54.

Upadhyaya, A., Davis, T. D. & Sankhla, N. (1986). Some biochemical changes associated with paclobutrazol-induced adventitious root formation on bean hypocotyl cuttings. *Ann. Bot.* 57:309-315.

van der Krieken, W. M., Breteler, H. & Visser, M. H. M. (1991). Indolebutyric acid-induced root formation in apple tissue culture -- Design of an experimental system auxin metabolism and isolation of cDNA clones related to root initiation. *Acta Hortic.* 289:343-344.

van der Krieken, W. M., Breteler, H. & Visser, M. H. M. (1992). Uptake and metabolism of indolebutyric acid during root formation on *Malus* microcuttings. *Acta Bot. Neerl.* 41:435-442.

van der Mast, C. A. (1970). The presence of membrane-bound IAA degrading protein-complex in homogenates of pea roots and the manner of attachment to these membranes. *Acta Bot. Neerl.* 19:553-566.

van der Salm, T. P. M., van der Toorn, C., Bouwer, R., Hänisch ten Cate, C. H. & Dons, H. J. M. (1997). Production of *ROL* gene transformed plants of *Rosa hybrida* L. and characterization of their rooting ability. *Mol. Breed.* 3:39-47.

van der Zaal, E. J., Groog, F. N., Boot, C. J., Hensgens, L. A., Hoge, J. H., Schilperoort, R. A. & Libbenga, K. R. (1991). Promoters of auxin-induced genes

from tobacco can lead to auxin-inducible and root-tip specific expression. *Plant Mol. Biol.* 16:983-998.

van Duijin, P. (1955). An improved histochemical benzidine-blue peroxidase method and a note on the composition of the blue reaction product. *Rev. Trav. Chim.* 74:771-778.

van Fleet, D. S. (1959). Analysis of the histochemical localization of peroxidase related to the differentiation of plant tissues. *Can. J. Bot.* 37:449-463.

van Staden, J. & Harty, A. R. (1988). Cytokinins and adventitious root formation. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation in Cuttings.* pp 185-201. Dioscorides Press. Portland, Oregon.

Vaughn, K. C. & Duke, S. O. (1984). Function of polyphenol oxidase in high plants. *Physiol. Plant.* 60:106-112.

Vaughn, K. C., Lax, A. R. & Duke, S. O. (1988). Polyphenol oxidase: the chloroplast oxidase with no established function. *Physiol. Plant.* 72:659-665.

Veierskov, B. (1988). Relations between carbohydrates and adventitious root formation. In Davis, T. D., Haissig, B. E. & Sankhla, N., eds. *Adventitious Root Formation by Cuttings.* pp 70-78. Dioscorides Press. Portland, Oregon.

Veierskov, B. & Andersen, A. S. (1982). Dynamics of extractable carbohydrates in *Pisum sativum.* III. The effect of IAA and temperature on content and translocation of carbohydrates in pea cuttings during rooting. *Physiol. Plant.* 55:179-182.

Veierskov, B., Andersen, A. S. & Eriksen, E. N. (1982). Dynamics of extractable carbohydrates in *Pisum sativum.* I. Carbohydrate and nitrogen content in pea plants and cuttings grown at two different irradiances. *Physiol. Plant.* 55:167-173.

Venverloo, C. J. (1976). The formation of adventitious organs. III. A comparison of root and shoot formation on *Nautilocalyx* explants. *Z. Pflanzenphysiol.* 80:310-322.

Vera, P., Lamb, C. & Doerner, P. W. (1994). Cell-cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene expression during the initiation of lateral root meristems. *Plant J.* 6:717-727.

Villalobos, V. M., Oliver, M. J., Yeung, E. C. & Thorpe, T. A. (1984). Cytokinin induced switch in development in excised cotyledons of radiata pine cultured *in vitro*. *Physiol. Plant.* 61:483-489.

Visser, E. J. W., Cohen, J. D., Barendse, G. W. M., Blom, C. W. P. M. & Voesenek, L. A. C. J. (1996). An ethylene-mediated increase in sensitivity to auxin induces adventitious root formation in flooded *Rumex palustris* Sm. *Plant Physiol.* 112:1687-1692.

Visser, E. J. W., Heijink, C. J., van Hout, K. J. G. M., Voesenek, L. A. C. J., Barendse, G. W. M. & Blom, C. W. P. M. (1995). Regulator role of auxin in

adventitious root formation in two species of *Rumex*, differing in their sensitivity to waterlogging. *Physiol. Plant.* 93:116-122.

Walter, C., Smith, D. R., Connett, M. B., Grace, L. & White, D. W. R. (1994). A biolistic approach for the transfer and expression of a *gus*A reporter gene in embryogenic cultures of *Pinus radiata*. *Plant Cell Reports* 14:69-74.

Wample, R. L. & Reid, D. M. (1979). The role of endogenous auxins and ethylene in the formation of adventitious roots and hypocotyl hypertrophy in flooded sunflower plants (*Helianthus annuus*). *Physiol. Plant.* 45:219-226.

Wang, A. M., Doyle, M. V. & Mark, D. F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 86:9717-9721.

Wang, Q. (1991). Factors affecting rooting of microcuttings of the pear rootstock BP10030. *Scientia Hortic.* 45:209-216.

Webb, J. & Jackson, M. B. (1986). A transmission and cryo-scanning electron microscope study of the formation of aerenchyma (cortical gas-filled space) in adventitious roots of rice (*Oryza sativa*). *J. Exp. Bot.* 37:832-841.

Webster, P. L. & Van't Hof, J. (1970). DNA synthesis and mitosis in meristems: requirements for RNA and protein synthesis. *Amer. J. Bot.* 57:130-139.

White, F., Ghidossi, G., Gordon, M. & Nester, E. (1982). Tumor induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the plant genome. *Proc. Natl. Acad. Sci. USA* 79:3193-3197.

White, F. F., Taylor, B. H., Huffman, G. D., Gordon, M. P. & Nester, E. W. (1985). Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *J. Bact.* 164:33-44.

White, J. & Lovell, P. H. (1984). The anatomy of root initiation in cuttings of *Griselinia littoralis* and *Griselinia lucida*. *Ann. Bot.* 54:7-20.

Wiesman, Z. & Lavee, S. (1994). Vegetative growth retardation, improved rooting and viability of olive cuttings in response to application of growth retardants. *Plant Growth Regul.* 14:83-90.

Wiesman, Z. & Lavee, S. (1995). Relationship of carbohydrate sources and indole-3-butyric acid in olive cuttings. *Aust. J. Plant Physiol.* 22:811-816.

Wilkie, S. (1997). Isolation of total genomic DNA. In Clark, M. S., ed. *Plant Molecular Biology - A Laboratory Mannual.* pp 3-15. Springer-Verlag. New York.

Willmitzer, L., Sanches-Serano, J., Buschfeld, E. & Schell, J. (1982). DNA from *Agrobacterium rhizogenes* is transferred to and expressed in axenic hairy root plant tissues. *Mol. Gen. Genet.* 186:16-22.

Wilson, C. L. (1927). Adventitious roots and shoots in an introduced weed. *Bull. Torrey Bot. Club* 54:35-38.

Wright, R. C. M. (1973). *A Handbook of Plant Propagation.* Ward Lock Limited. London.

Wu, F. T. & Barnes, M. F. (1981). The hormonal levels in stem cuttings of difficult-to-root and easy-to-root rhododendrons. *Biochem. Physiol. Pflanzen* 176:13-22.

Zaenen, I., van larebeke, N., Teuchy, H., van Montagu, M. & Schell, J. (1974). Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. *J. Mol. Biol.* 86:109-127.

Zimmerman, R. H. (1984). Rooting apple cultivars *in vitro.* Interactions among light, temperature, phloroglucinol and auxin. *Plant Cell Tiss. Org. Cult.* 3:301-311.

Zimmerman, R. H. & Broome, O. (1981). Phloroglucinol and *in vitro* rooting of apple cultivar cuttings. *J. Amer. Soc. Hort. Sci.* 106:648-652.

Zurfluh, L. L. & Guilfoyle, T. J. (1982). Auxin- and ethylene-induced changes in the population of translatable messenger RNA in basal sections and intact soybean hypocotyl. *Plant Physiol.* 69:338-340.

Appendix A

1. *2D-MH extraction buffer*

2% (v:v) 2D Pharmalyte pH 3-10; 0.3 M NaCl; 1 mM EDTA-Na; 1 mM EGTA; 1% (v:v) Nonidet P-40; 1% (w:v) CHAPS; 5 mM ascorbic acid; 0.1 M DTT; 10 μg/ml leupeptin; 10 μg/ml α_2-macroglobulin

2. *Separating gel solution (75 ml for 2 gels)*

30 ml Acrylamide stock (29.2% acrylamide and 0.8% Bis); 26.25 ml ddH_2O; 18.75 ml separating gel buffer (1.5M Tris-HCl, pH 8.8, 0.4% SDS); 45 μl TEMED; 450 μl 10% ammonium persulfate

3. *Stacking gel solution (30 ml for 2 gels)*

4.8 ml Acrylamide stock (29.8% acrylamide and 0.8% Bis); 17.7 ml ddH_2O; 7.5 ml separating gel buffer (0.5M Tris-HCl, pH 6.8, 0.4% SDS); 30 μl TEMED; 75 μl 10% ammonium persulfate

4. *2× SDS sample buffer*

To 40 ml dH_2O add: 1.52 g Tris base; 20 ml glycerol; 2.0 g SDS; 1 mg Bromphenol blue, adjust to pH 6.8 with 1 N HCl, add d H_2O to 100 ml

5. *Electrode buffer*

Dissolve 57.6 g glycine; 12 g Tris base; 2 g SDS; 0.4 g sodium azide (lower buffer only) into 2 litre d H_2O.

6. *IEF gel solution*

10 g urea; 2.5 ml acrylamide/PDA (30/0.8%); 7 ml dH_2O; 0.2 ml Resolyte pH 4-8; 0.8 ml 2D Pharmalyte pH 3-10; *0.3 g CHAPS; *0.1 ml Nonidet P-40 (* added as a combined solution, 0.3g CHAPS; 0.1 ml Nonidet P-40; 0.9 ml dH_2O)

7. *Ammonia silver nitrate solution*

6 g of silver nitrate was dissolved in 30 ml dH_2O. 6 ml of this solution was dropwisely mixed into a solution containing 30.6 ml of 0.36% NaOH and 2.28 ml of NH_4OH. To this, 110 ml of dH_2O was slowly added to this solution with constant stirring.

8. *Dinitrosalicylic acid colour reagent*

Dissolve at room temperature 1 g of 3,5-dinitrosalicylic acid in 20 ml of 2 N NaOH and 50 ml distilled water, add 30 g of Rochelle salt to 100 ml with distilled water, only prepared freshly before use.

9. *5× reaction buffer for terminal transferase (BOEHRINGER MANNHEIM)*

1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine albumin, pH 6.6 at 25°C

10. *TE buffer (Tris/EDTA)*

10 mM Tris-HCl pH 7.4, 0.1 mM EDTA

11. *TES buffer (Tris/EDTA/sodium chloride)*

10 mM Tris-HCl pH7.4, 1 mM EDTA, 100 mM NaCl

12. *10× ligation buffer (TA Cloning® Kit, Invitrogen®)*

60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM DTT, and 10 mM spermidine

13. SOC medium

2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose.

1) For 1 litre, dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water; 2) add 10 ml of 250 mM KCl to the solution in the above; 3) adjust pH to 7.0 with 5 M NaOH, then bring the volume to 980 ml with deionized water; 4) prepare a 1 M $MgCl_2$ solution; 5) autoclave both solutions; 6) meanwhile, make a 2 M glucose solution and filter-sterilized; 7) let the autoclaved solution cool to about 55°C, then add 10 ml of the filter-sterilized 2 M glucose solution and 10 ml of 1 M $MgCl_2$. Store at room temperature or +4°C.

14. LB (Luria-Bertani) medium

1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0. Add agar to 1.5% if solid medium is used

15. X-gal solution

40 mg of X-gal in 1 ml of dimethylformamide (DMF)

16. DNA Loading buffer (Type III)

0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water

17. TAE buffer

0.04 M Tris-acetate, 0.001 M EDTA (to make up 1 litre of 50× stock: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA(pH 8.0))

18. RNA dilution buffer

Mix DEPC-treated water: 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0): formaldehyde (5:3:2)

19. Pre-hybridization solution (high SDS hybridization buffer)

7% SDS, 50% formamide (deionized), 5 × SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 2% blocking reagent (*BOEHRINGER MANNHEIM*), 50 mM sodium-phosphate (pH 7.0), and 1% N-lauroylsarcosine

20. 2 × wash solution

2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) containing 0.1% SDS

21. 0.1 × wash solution

0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) containing 0.1% SDS

22. Washing buffer

maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5), 3% (v/v) Tween 20

23. Blocking solution

1% (w/v) blocking reagent (*BOEHRINGER MANNHEIM*) in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5)

24. Antibody solution

Dilute the Anti-Digoxigenin-AP (*BOEHRINGER MANNHEIM*, 750 units/ml) at 1:5000 in blocking solution

25. Detection buffer

100 mM Tris-HCl, 100 mM NaCl, pH 9.5

26. Colour substrate solution

Add 100 µl of NBT/BCIP solution (*BOEHRINGER MANNHEIM*) to 5 ml of detection buffer

Appendix B

The behaviour of proteins separated by 2D-PAGE

NO.	Time (days after excision)											NO.	Time (days after excision)										
		IBA treatment					Control							IBA treatment					Control				
	0	1	4	7	10	13	1	4	7	10	13		0	1	4	7	10	13	1	4	7	10	13
1	+	+	+	+	+		+					42				+							
2	+	+	+	+			+	+	+	+		43					+	+					
3	+	+			+		+					44	+	+	+								
4	+											45	+	+	+	+	+	+	+	+	+	+	+
5	+											46	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+		+		+	+	+	+	+	47	+	+	+	+	+	+	+	+	+	+	+
7	+	+							+	+		48	+	+					+				
8	+											49	+										
9	+				+	+					+	50	+	+									
10	+											51	+	+						+			
11	+	+	+				+	+	+	+	+	52	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+				+	+	+	+	+	53	+	+	+				+	+	+	+	+
13	+	+	+	+	+	+	+	+				54	+	+		+							
14	+			+	+							55	+			+	+	+	+	+	+	+	
15	+	+	+	+	+	+	+	+	+	+	+	56	+	+	+								
16	+	+					+	+	+	+	+	57	+	+	+					+			+
17	+	+	+				+	+	+	+	+	58	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+				+	+	+	+		59	+	+	+	+	+	+	+	+	+	+	+
19	+											60	+	+					+	+	+	+	
20	+	+					+					61	+						+				
21	+	+	+	+	+	+	+	+	+	+	+	62	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+					63	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+		+					64	+	+	+	+	+	+	+	+	+		
24	+	+	+	+	+	+	+	+	+	+	+	65	+	+	+	+			+	+	+	+	+
25	+	+						+				66	+	+	+	+	+	+	+	+	+	+	+
26	+	+					+	+	+	+		67	+	+									
27	+			+	+	+	+				+	68	+						+	+	+	+	+
28	+	+	+	+	+	+	+	+	+	+	+	69	+	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	+	70	+	+	+				+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	71	+	+	+				+				
31	+	+	+	+	+	+	+	+	+	+	+	72	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	73	+	+	+	+	+	+	+	+	+	+	+
33	+	+										74	+	+	+				+	+	+	+	+
34	+	+	+				+	+	+	+	+	75										+	+
35	+	+					+					76	+	+					+	+	+	+	+
36	+	+	+				+	+	+	+	+	77	+	+	+				+	+			
37	+	+		+								78	+	+	+	+	+	+	+	+	+	+	+
38		+	+		+							79	+						+	+	+	+	+
39			+	+	+		+	+	+	+	+	80	+	+					+				
40			+	+	+	+		+	+	+	+	81	+	+					+				
41			+	+	+	+						82	+	+	+	+	+	+	+	+	+	+	+

Appendix B continued

NO.	Time (days after excision)											NO.	Time (days after excision)										
		IBA treatment					Control							IBA treatment					Control				
	0	1	4	7	10	13	1	4	7	10	13		0	1	4	7	10	13	1	4	7	10	13
83	+	+	+	+	+	+	+	+	+	+	+	163	+	+	+	+	+	+	+	+	+	+	+
84	+						+	+	+	+	+	164	+	+	+	+	+	+	+	+	+	+	+
85	+	+					+	+	+			165	+	+	+	+	+						
86	+	+	+									166	+			+	+				+		
87	+		+				+	+	+	+		167	+	+	+	+	+	+	+	+	+	+	+
88	+	+	+	+			+	+	+	+		168	+	+	+				+	+	+	+	
89	+	+	+	+	+		+					169	+	+		+	+	+			+		
90	+			+							+	170	+		+	+	+	+	+	+	+	+	+
91	+	+	+	+	+	+	+	+	+	+	+	171	+	+	+	+	+	+	+	+	+	+	+
92	+	+	+	+	+	+	+	+	+	+	+	172	+	+									
93	+	+	+				+	+	+	+		173	+	+	+	+	+	+	+	+	+	+	+
94	+	+	+	+	+		+	+	+	+		174	+	+	+	+	+	+	+				
95	+	+	+	+	+		+	+	+			175	+	+	+	+	+	+	+	+	+	+	+
96	+	+	+				+				+	176	+	+	+	+	+	+	+	+	+	+	+
97		+	+				+	+	+	+	+	177	+	+	+	+	+	+	+	+	+	+	+
98							+	+	+	+	+	178		+	+				+	+			
99		+					+					179	+	+	+	+	+		+				
100		+				+		+	+			180	+			+							
101		+	+	+	+	+	+	+	+	+	+	181	+	+	+	+			+				+
102			+	+	+	+		+	+	+		182	+	+	+	+	+	+	+	+	+	+	+
103			+	+	+	+						183	+	+	+	+	+	+	+	+	+	+	+
104				+	+	+						184	+	+	+	+	+	+	+	+	+		
105				+	+	+						185	+						+	+			
106							+	+	+	+	+	186	+	+	+				+	+			
107								+	+			187	+	+	+	+	+	+	+	+	+	+	+
108								+	+			188	+	+	+	+	+	+	+	+	+	+	+
133	+	+	+	+	+	+	+	+	+	+	+	189	+	+	+	+	+	+	+	+	+	+	+
134	+	+	+	+	+	+	+	+	+	+	+	190	+	+	+	+	+	+	+	+	+	+	+
135	+	+	+	+	+	+	+	+	+	+	+	191	+										
136	+	+	+	+	+	+	+	+	+	+	+	192	+	+	+	+	+	+	+	+	+	+	+
137	+	+	+									193	+	+	+	+	+	+	+	+	+	+	+
138	+	+	+	+			+	+			+	194	+	+	+	+				+			+
139	+	+	+				+	+	+	+		195	+	+	+						+	+	+
140	+	+	+					+		+		196	+	+		+	+	+	+	+	+		
141	+			+	+		+	+	+	+		197	+								+		
142	+											198	+		+								
143	+		+	+			+	+				199	+	+	+	+	+	+	+	+	+	+	+
144	+	+	+	+	+	+	+	+	+	+	+	200	+										
145	+	+	+	+	+	+	+	+	+	+	+	201		+	+	+	+		+				
146	+	+	+				+	+	+	+		202		+	+	+							
147	+	+	+	+	+	+	+	+	+	+	+	203		+	+	+	+						
148	+	+	+	+	+	+	+	+	+	+	+	204	+	+	+								
149	+	+	+	+	+	+	+	+	+	+	+	205		+	+	+	+	+	+	+	+	+	+
150	+	+	+	+	+	+	+	+	+	+	+	206			+	+	+	+				+	+
151	+	+	+	+	+	+	+	+				207		+	+	+	+	+	+	+	+	+	+
152	+	+	+	+			+	+	+	+	+	208			+	+				+	+	+	
153	+	+	+	+	+	+	+	+	+	+	+	209			+	+	+	+		+	+	+	+
154	+	+	+	+	+	+	+	+	+	+	+	210			+	+	+	+					+
155	+	+	+			+	+	+	+	+	+	211				+	+	+					
156	+	+	+	+	+	+	+	+	+	+	+	212				+	+	+					
157	+	+	+	+	+	+		+	+	+	+	213				+	+						
158	+	+			+	+		+	+	+	+	214				+	+			+	+	+	
159	+	+								+		215	+	+	+			+	+	+			+
160	+	+	+	+			+	+	+	+	+	216	+	+	+	+	+	+	+	+	+	+	+
161	+	+	+	+	+	+	+	+	+	+	+	217	+				+		+	+	+	+	+
162	+	+	+	+			+	+	+	+		218	+	+	+	+	+	+	+	+	+	+	+

Appendix B continued

NO.	Time (days after excision)											NO.	Time (days after excision)									
		IBA treatment					Control							IBA treatment					Control				
	0	1	4	7	10	13	1	4	7	10	13		0	1	4	7	10	13	1	4	7	10	13
219	+		+	+	+	+						163	+	+	+	+	+	+	+	+	+	+	+
220	+		+	+								164	+	+	+	+	+	+	+	+	+	+	+
221	+	+	+	+	+	+	+	+	+	+	+	165	+	+	+	+	+						
222	+	+	+	+	+	+	+	+	+			166	+			+	+				+		
223	+	+	+	+			+	+	+		+	167	+	+	+	+	+	+	+	+	+	+	+
224	+	+	+	+	+	+	+	+	+	+	+	168	+	+	+				+	+	+	+	
111	+	+	+	+			+	+	+			169	+	+		+	+	+			+		
112	+	+	+									170	+		+	+	+	+	+	+	+	+	+
113	+	+	+	+	+	+	+	+	+	+	+	171	+	+	+	+	+	+	+	+	+	+	+
114	+	+	+	+			+	+	+	+	+	172	+	+									
115	+	+	+	+								173	+	+	+	+	+	+	+	+	+	+	+
116	+		+	+								174	+	+	+	+	+	+	+				
117	+		+	+								175	+	+	+	+	+	+	+	+	+	+	+
118	+	+	+	+	+	+						176	+	+	+	+	+	+	+	+	+	+	+
119	+											177	+	+	+	+	+	+	+	+	+	+	+
120	+	+										178		+	+				+	+			
121	+	+										179	+	+	+	+	+		+				
122	+											180	+			+							
123	+			+			+					181	+	+	+	+			+				+
124	+											182	+	+	+	+	+	+	+	+	+	+	+
125	+											183	+	+	+	+	+	+	+	+	+	+	+
126	+	+	+	+	+	+	+	+	+	+	+	184	+	+	+	+	+	+	+	+	+		
127	+	+	+	+	+	+	+	+	+	+	+	185	+						+	+			
128	+	+	+	+	+	+	+	+	+	+	+	186	+	+	+				+	+			
129	+	+	+	+	+	+	+	+	+	+	+	187	+	+	+	+	+	+	+	+	+	+	+
130	+	+										188	+	+	+	+	+	+	+	+	+	+	+
131	+	+										189	+	+	+	+	+	+	+	+	+	+	+
132	+	+	+	+	+	+	+	+	+	+	+	190	+	+	+	+	+	+	+	+	+	+	+
133	+	+	+	+	+	+	+	+	+	+	+	191	+										
134	+	+	+	+	+	+	+	+	+	+	+	192	+	+	+	+	+	+	+	+	+	+	+
135	+	+	+	+	+	+	+	+	+	+	+	193	+	+	+	+	+	+	+	+	+	+	+
136	+	+	+	+	+	+	+	+	+	+	+	194	+	+	+	+				+			+
137	+	+	+									195	+	+	+						+	+	+
138	+	+	+	+			+	+			+	196	+	+		+	+	+	+	+	+		
139	+	+	+				+	+	+	+		197	+								+		
140	+	+	+					+		+		198	+		+								
141	+			+	+		+	+	+	+		199	+	+	+	+	+	+	+	+	+	+	+
142	+											200	+										
143	+		+	+			+	+				201		+	+	+	+		+				
144	+	+	+	+	+	+	+	+	+	+	+	202		+	+	+							
145	+	+	+	+	+	+	+	+	+	+	+	203		+	+	+	+						
146	+	+	+				+	+	+	+		204	+	+	+								
147	+	+	+	+	+	+	+	+	+	+	+	205		+	+	+	+	+	+	+	+	+	+
148	+	+	+	+	+	+	+	+	+	+	+	206			+	+	+	+				+	+
149	+	+	+	+	+	+	+	+	+	+	+	207		+	+	+	+	+	+	+	+	+	+
150	+	+	+	+	+	+	+	+	+	+	+	208			+	+				+	+	+	
151	+	+	+	+	+	+	+	+				209			+	+	+	+		+	+	+	+
152	+	+	+	+			+	+	+	+	+	210			+	+	+	+					+
153	+	+	+	+	+	+	+	+	+	+	+	211				+	+	+					
154	+	+	+	+	+	+	+	+	+	+	+	212				+	+	+					
155	+	+	+			+	+	+	+	+	+	213				+	+						
156	+	+	+	+	+	+	+	+	+	+	+	214				+	+			+	+	+	
157	+	+	+	+	+	+		+	+	+	+	215	+	+	+			+	+	+			+
158	+	+			+	+		+	+	+	+	216	+	+	+	+	+	+	+	+	+	+	+
159	+	+								+		217	+				+		+	+	+	+	+
160	+	+	+	+			+	+	+	+	+	218	+	+	+	+	+	+	+	+	+	+	+
161	+	+	+	+	+	+	+	+	+	+	+	219	+		+	+	+	+					
162	+	+	+	+			+	+	+	+		220	+		+	+							

Appendix B continued

NO.	Time (days after excision)											NO.	Time (days after excision)										
		IBA treatment					Control							IBA treatment					Control				
	0	1	4	7	10	13	1	4	7	10	13		0	1	4	7	10	13	1	4	7	10	13
221	+	+	+	+	+	+	+	+	+	+	+	279	+	+	+	+	+	+	+	+	+	+	+
222	+	+	+	+	+	+	+	+	+			280	+	+	+	+			+	+	+	+	+
223	+	+	+	+			+	+	+		+	281	+	+	+		+	+	+	+	+	+	+
224	+	+	+	+	+	+	+	+	+	+	+	282	+	+	+			+		+	+		
225	+	+	+									283	+	+	+	+	+	+	+	+	+	+	+
226	+	+	+		+		+	+			+	284	+	+	+	+	+	+	+	+	+	+	+
227	+		+	+				+		+		285	+	+	+	+	+	+	+	+	+	+	+
228	+		+	+	+	+						286	+	+	+				+	+	+	+	+
229	+	+	+	+	+	+						287	+	+	+				+	+	+	+	+
230	+		+	+	+	+						288	+	+					+	+	+	+	+
231	+	+	+	+								289	+	+	+				+	+	+	+	+
232	+		+	+			+	+	+			290	+	+	+	+	+	+	+	+	+	+	+
233	+	+	+	+	+	+	+	+	+	+	+	291	+	+	+	+	+	+	+	+	+	+	+
234	+	+	+	+	+	+	+	+	+	+	+	292	+	+	+	+	+	+	+	+	+	+	+
235	+	+	+	+	+	+	+	+	+	+	+	293	+	+	+			+	+	+	+	+	+
236	+	+	+	+	+	+	+	+	+	+	+	294	+	+	+	+	+	+	+	+	+	+	+
237	+	+	+	+	+	+	+	+	+	+	+	295	+	+	+	+	+	+	+	+	+	+	+
238	+	+	+		+	+	+	+	+	+	+	296	+	+	+	+	+	+	+	+	+	+	+
239	+											297	+	+	+	+	+	+	+	+	+	+	+
240	+	+	+	+	+	+	+	+				298	+	+					+	+	+		
241	+	+	+	+	+	+	+	+	+	+	+	299	+	+	+	+	+	+	+	+	+	+	+
242	+	+	+	+	+	+	+	+	+	+	+	300	+						+	+		+	
243	+	+	+	+	+	+	+	+	+	+	+	301	+	+	+	+	+			+	+	+	
244	+	+	+	+	+	+	+	+	+	+	+	302	+	+	+	+			+	+	+	+	+
245	+	+	+	+	+	+	+	+	+	+	+	303	+	+	+	+	+	+	+	+	+	+	+
246	+											304	+	+	+	+	+	+	+	+	+	+	+
247	+	+					+	+				305	+	+	+	+	+	+	+	+	+	+	+
248	+	+	+	+	+	+	+	+	+	+	+	306	+	+	+	+	+	+	+	+	+	+	+
249	+	+	+	+	+	+	+	+	+	+	+	307	+	+	+	+		+		+	+	+	+
250	+	+	+	+	+	+	+	+	+	+	+	308	+	+	+	+		+	+	+	+	+	
251	+	+	+		+		+	+	+	+		309	+	+	+			+	+	+	+	+	+
252	+	+	+	+	+	+	+	+	+			310	+	+		+		+	+		+	+	+
253	+	+	+	+	+	+	+	+	+	+	+	311	+	+	+	+			+	+	+		+
254	+	+	+	+	+		+	+	+			312	+	+					+		+	+	
255	+		+	+	+	+	+	+	+	+	+	313	+	+	+								
256	+	+	+	+	+	+	+	+	+	+	+	314	+	+	+								
257	+	+	+				+	+				315	+	+	+	+	+						
258	+	+	+	+	+	+	+	+	+	+	+	316	+	+	+	+	+			+	+	+	+
259	+	+	+				+					317	+									+	+
260	+			+			+	+	+	+		318	+									+	+
261	+						+	+	+	+		319	+			+							
262	+	+	+	+	+	+	+	+	+	+	+	320	+		+	+				+	+	+	
263	+	+	+	+	+	+	+	+	+	+	+	321	+	+	+	+	+	+	+	+	+	+	+
264	+		+	+	+	+	+	+	+	+	+	322	+	+	+	+	+	+		+	+		+
265	+	+	+	+	+	+	+	+	+	+	+	323	+	+	+			+					
266	+	+	+	+	+	+	+	+	+	+	+	324	+	+	+	+		+		+	+	+	+
267	+	+	+	+	+	+	+	+	+	+	+	325			+	+	+	+		+	+	+	+
268	+	+	+	+	+	+	+	+	+	+	+	326		+	+	+							
269	+	+	+	+	+	+	+	+	+	+	+	327		+	+	+	+	+	+	+	+	+	+
270	+	+	+				+	+	+			328			+	+							
271	+	+	+				+					329			+	+							
272	+	+	+							+		330			+	+	+	+		+			
273	+	+					+	+	+			331			+					+			
274	+	+	+	+	+	+	+	+	+			332			+					+			
275	+	+	+	+	+	+	+	+	+	+	+	333				+							
276	+	+	+	+	+	+	+	+	+	+	+	334				+	+						
277	+		+						+		+	335				+	+	+					
278	+	+	+		+	+		+	+			336					+						